



**Evaluating the Suitability of Waste Substrates
for Microalgal Biomass Production Using
Different Modes of Cultivation**

This work is submitted in fulfilment of the requirements of the degree of Master of Applied Sciences: Biotechnology in the Faculty of Applied Sciences at the Durban University of Technology.

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DECLARATION BY STUDENT

**Evaluating the Suitability of Waste Substrates for
Microalgal Biomass Production Using Different
Modes of Cultivation**

Prathana Ramsundar
2017

I declare that the thesis herewith submitted for the MAppSci: Biotechnology at the Durban University of Technology has not been previously submitted for a degree at any other University.

Prathana Ramsundar

REFERENCE DECLARATION

I, Prathana Ramsundar and Prof. Faizal Bux do hereby declare that in respect to the following dissertation: Evaluating the suitability of waste substrates for microalgal biomass production using different modes of cultivation. As far as we know and can as certain: no other similar dissertation exists: all references as detailed in the dissertation are complete in terms of all personal communications engaged in and published works consulted.

Signature of Student

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APPROVAL

I hereby approve the final submission of the following thesis.

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This _____ day _____ of 2017, at the Durban University of Technology.

ABSTRACT

The utilization of wastewater as a substrate for microalgal biomass cultivation is one of the few potentially viable routes for fuel and feed applications. In this study, the suitability of various liquid wastewater streams and waste biosolids from a domestic wastewater treatment plant was assessed for microalgal cultivation. The wastewater substrates were analyzed for nutrient content as a potential growth medium. For liquid waste substrates, physical, thermal and biological pre-treatment methods were evaluated to minimize the bacterial load. Biomass, physiology, nutrient removal efficiencies and biochemical constituents of *Chlorella sorokiniana* were investigated in influent (INF) and anaerobic tank centrate (AC) under mixotrophic (Mixo) and heterotrophic (Hetero) cultivation modes. Mixotrophic cultivation conditions demonstrated efficient ammonium (94.29%) and phosphate (83.30%) removal with promising biomass ($77.14 \text{ mgL}^{-1}\text{d}^{-1}$), lipid ($24.91 \text{ mgL}^{-1}\text{d}^{-1}$), protein ($22.36 \text{ mgL}^{-1}\text{d}^{-1}$) and carbohydrate ($20.10 \text{ mgL}^{-1}\text{d}^{-1}$) productivities. Urea supplementation (1500 mgL^{-1}) further enhanced biomass ($162.50 \text{ mgL}^{-1}\text{d}^{-1}$), lipid ($24.91 \text{ mgL}^{-1}\text{d}^{-1}$), protein ($22.36 \text{ mgL}^{-1}\text{d}^{-1}$) and carbohydrate ($20.10 \text{ mgL}^{-1}\text{d}^{-1}$) productivities in Mixo AC. Therefore, the urea supplemented Mixo AC approach for microalgal cultivation was developed as a suitable biomass production strategy.

This work also elucidated a novel algae cultivation strategy for utilisation of waste biosolids, where nutrient-rich waste activated sludge (WAS) and final effluent (FE) from the wastewater treatment process was used for microalgal biomass generation. This strategy reduced the use of synthetic nutrients, fertilizers and freshwater which contribute significantly towards the overall cost of biomass production. Strategy development included the

investigation of physical, thermal and chemical pre-treatment methods to assist in effective nutrient release and bacterial load reduction. Evaluation of growth kinetics, photosynthetic performance, nutrient removal efficiencies and biochemical composition of microalgae under mixotrophic and heterotrophic modes of cultivation were performed. Furthermore, urea supplementation was studied to improve biomass productivity. Microalgae cultivation in acid pre-treated (pH 2) WAS + FE with urea supplementation (1500 mgL^{-1}) showed enhanced biomass productivity of $298.75 \text{ mgL}^{-1}\text{d}^{-1}$. Microalgal biomass grown with WAS + FE using the developed strategy exhibited greater lipid ($72.95 \text{ mgL}^{-1}\text{d}^{-1}$) and protein ($72.84 \text{ mgL}^{-1}\text{d}^{-1}$) productivities and comparable carbohydrate yields ($73.07 \text{ mgL}^{-1}\text{d}^{-1}$) to that of synthetic media. Thus mixotrophic mode of cultivation coupled with urea supplementation to WAS + FE proved to be a suitable cultivation strategy for *C. sorokiniana*.

The study developed an efficient strategy to utilize AC and WAS + FE as a growth medium for microalgae. Furthermore, findings from this study have demonstrated the potential of waste streams and waste solids from domestic wastewater treatment plants for microalgal biomass generation.

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DEDICATION

This thesis is dedicated to my beloved parents, Udesb Ramsundar and Usha Ramsundar.

Thank you for being my driving force and inspiration.

PREFACE

The research outputs of the Masters qualification are as follows:

Published articles in accredited journals:

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Chlorophyll *a*; Chl-*a* ($\mu\text{g mL}^{-1}$) = $16.72 A_{665.2} - 9.16 A_{652.4}$ (1).....33

Chlorophyll *b*; Chl-*b* ($\mu\text{g mL}^{-1}$) = $34.09 A_{652.4} - 15.28 A_{665.2}$ (2).....33

$rETR = F'_q / F'_m \times PPF D$ (3)34

$F_v/F_m = F_m - F_o / F_m$ (4)34

LIST OF ABBREVIATIONS

AC – anaerobic tank centrate	Hetero – heterotrophic cultivation
ANOVA – analysis of variance	INF – final effluent post chlorination
ATP – adenosine triphosphate	IR – infrared spectroscopy
Auto – autotrophic cultivation mode	IP – inorganic phosphorous
BG11 – blue-green medium	K – potassium
BNR – biological nutrient removal	MAE – microwave assisted extraction
BOD – biological oxygen demand	Mg – magnesium
BSA – bovine serum albumin	Mixo – mixotrophic cultivation
Ca – calcium	Mixo AC 500 – mixotrophic cultivation of anaerobic tank centrate + 500 mgL ⁻¹ urea
Chl – chlorophyll	Mixo AC 1500 – mixotrophic cultivation of anaerobic tank centrate + 1500 mgL ⁻¹ urea
CO ₂ – carbon dioxide	Mn - manganese
Co – cobalt	Mo – molybdate
COD – chemical oxygen demand	MP-AES – Microwave Plasma-Atomic Emission spectroscopy
Cu – copper	MV – microwave heating
DCW – dry cell weight	N – nitrogen
ETR – electron transport rate	Na – sodium
FAO – Food and Agricultural Organization	NAIP – non-apatite inorganic phosphorous
FE – final effluent	NaOH – sodium hydroxide
Fe – iron	NADH – nicotinamide adenine dinucleotide
G3P – glyceraldehyde-3-phosphate	NH ₄ – ammonium
HCl – hydrochloric acid	NH ₄ MgPO ₄ .6H ₂ O - struvite
HPC – heterotrophic plate count	NMR – Nuclear Magnetic Resonance

NO ₂ – nitrite	SO ₄ – sulphur
NO ₃ – nitrate	TDS – total dissolved solids
NTP – nitrogen protein conversion factor	TN – total nitrogen
OD – optical density	TP – total phosphorous
OP – organic phosphorous	TS – total solids
P – phosphorous	TSS – total suspended solids
PAM – Pulse Amplitude Modulation	UAE – ultrasound assisted extraction
Fluorometry	
PAR – photosynthetically active radiation	UK – United Kingdom
PGA – 3-phosphoglyceraldehyde	USA – United States of America
PO ₄ – phosphate	UV-vis spectroscopy – Ultraviolet-visible spectroscopy
PPFD – photosynthetically active photon flux density	WAS – waste activated sludge
PS II – photosystem II	WAS + FE – waste activated sludge in final effluent
R-2A agar – Reasoner’s 2A agar	WAS + FE 250 – treated waste activated sludge in final effluent + 250 mgL ⁻¹ urea
RASL – return activated sludge liquor	WAS + FE 500 – treated waste activated sludge in final effluent + 500 mgL ⁻¹ urea
RAW – untreated waste activated sludge in final effluent	WAS + FE 1500 – treated waste activated sludge in final effluent + 1500 mgL ⁻¹ urea
rETR – relative electron transport rate	WHO – World Health Organization
RuBisCO – Ribulose-1,5-bisphosphate carboxylase/oxygenase	WWTP – wastewater treatment plant
RuBP – Ribulose-1,5-biphosphate	Zn - zinc
S.A. – South America	

LIST OF MATHEMATICAL SYMBOLS

Beta – β	Micromole – μmol
Colony forming units per 100 mL – CFU 100 mL ⁻¹	Milligram – mg
Colony forming units per mL – CFU mL ⁻¹	Milligrams per litre – mgL ⁻¹
Degrees celcius – °C	Milligrams per litre per day – mgL ⁻¹ d ⁻¹
Euro per kilogram – € kg ⁻¹	Millijoule per square centimetre – mJ cm ⁻²
Grams – g	Millilitre – mL
Grams per litre – gL ⁻¹	Minutes – min
Gram per dry cell weight – g ⁻¹ DCW	Molar – M
Grams per litre per day – gL ⁻¹ d ⁻¹	Most Probable Number per litre – MPN L ⁻¹
Hours – h	Nano meter – nm
Intrinsic fluorescence – F _o	Nephelometric Turbidity Unit – NTU
Kilowatt hour per kilogram – kWh kg ⁻¹	Percent – %
Litre – L	Per day – d ⁻¹
Maximum efficiency of PS II – F _v /F _m	Percentage by weight – wt. %
Maximal fluorescence yield – F _m	PS II operating efficiency – F' _q /F' _m
Maximum fluorescence in a light adapted sample – F' _m	Relative centrifugal force – × g
Metres per second – m ² s ⁻¹	Rotations per minute – rpm
Microgram per millilitre - $\mu\text{g mL}^{-1}$	Seconds – s
Microlitre – μL	Standard deviation – SD
Micrometre – μm	Variable fluorescence – F _v
Wattage in watts – W	

CHAPTER ONE

INTRODUCTION

1.1. Context of Research

Microalgal biomass is considered to be a sustainable feedstock for the production of biofuels and several other co-products, therefore research on the cultivation of microalgae is rapidly emerging (Lam and Lee, 2013). Microalgae have shown high growth rates and environmental benefits that include nutrient recovery and CO₂ uptake (Cai *et al.*, 2013, Li *et al.*, 2008b). The biochemical constituents such as lipids, carbohydrates, proteins and other bioactive compounds derived from microalgae have various potential commercial applications in the biofuels, animal feed, nutraceuticals and pharmaceuticals industries (Guo *et al.*, 2013, Singh *et al.*, 2015). However, the high cost of microalgal biomass production is the major bottleneck in commercial realization of commodity products such as biofuels or feed additives (Ren *et al.*, 2014).

Conventional chemical components (nitrates, phosphates and glucose) and large amounts of water are major contributors to high microalgal cultivation costs. Recently nutrient-rich waste substrates such as wastewaters are being investigated as alternate nutrient and water sources for sustainable microalgal biomass production (Li *et al.*, 2011a, Wang *et al.*, 2010b, Zhou *et al.*, 2012b). In domestic wastewater treatment process various waste streams are generated such as effluent after primary screening, secondary effluent, final effluent, anaerobic centrate. Streams such as anaerobic centrate are sent back to the head of the treatment works for further treatment. Final effluent is discharged into the rivers and other water bodies.

Whereas, waste products such as sludge (solid fraction of the wastewater) from the activated sludge treatment process are generated in massive quantities and are problematic during disposal. In order to achieve efficient waste management and prevent environmental hazards, a suitable use for these wastes needs to be established.

The waste streams and solids generally contain significant amounts of nitrogen (N), phosphorous (P) and organic carbon (C) that can be exploited as nutrient sources for microalgal cultivation (Liang, 2013). Recently these wastes streams and by-products of the wastewater treatment process have been gaining interest of the researchers as nutrient source for microalgal cultivation to replace or minimize the use of conventional chemical nutrients (Cabanelas *et al.*, 2013b, Lizzul *et al.*, 2014, Mutanda *et al.*, 2011). However, each waste stream has various challenges including nutrient availability and bacterial contamination. Thus individual streams have to be extensively evaluated for the suitability of microalgal cultivation.

Microalgae are able to thrive in different modes of cultivation (photoautotrophic, heterotrophic and mixotrophic) by adopting various routes of metabolism for their growth and survival (Prathima Devi *et al.*, 2012). Photoautotrophic microalgae are the most predominant algae in nature and are commonly cultured commercially in open ponds and photobioreactors with the supply of CO₂ or air under either natural sunlight or artificial illumination and synthetic media. In heterotrophic mode microalgae is not dependent on the light for energy and utilizes organic carbon as energy source. In mixotrophic mode microalgae use both light and organic carbon as energy sources. The composition of waste materials strongly impacts the mode of cultivation. Studies using waste substances as substrates for microalgal growth have been carried out using conventional photoautotrophic cultivation (Akerstrom *et al.*, 2014,

Gentili, 2014). However, mixotrophic and heterotrophic cultivation in waste substrates have not been extensively researched.

The waste substrates could be applied as nutrient sources for economical microalgal cultivation. However there are challenges such as nutrient availability, low biomass productivities and bacterial contamination which need to be addressed. This study thus focuses on addressing these challenges and developing suitable cultivation strategies using waste substrates.

1.2. Aim and Objectives of Study

1.2.1. Aim

To evaluate the suitability of waste substrates for microalgal biomass production using different modes of cultivation.

1.2.2. Objectives

- To evaluate the nutrients availability in waste substrates for microalgal cultivation by physico-chemical characterization.
- To investigate the pre-treatment methods for nutrient release and removal of microbial contamination from waste substrates for efficient microalgal cultivation.
- To analyze growth and physiology of microalgal culture grown on selected waste substrates using different modes of cultivation.

- To compare microalgal cultivation on selected waste substrates and synthetic media by analyzing growth physiology and biomass production.
- To biochemically characterize microalgal biomass to determine their potential applications in the fuel and feed industries.

CHAPTER TWO

LITERATURE REVIEW

GULDHE, A., KUMARI, S., RAMANNA, L., RAMSUNDAR, P., SINGH, P., RAWAT, I., BUX, F. 2017. Prospects, recent advancements and challenges of different wastewater streams for microalgal cultivation. *Journal of Environmental Management*, 203, 299–315.

2.1. Introduction

Microalgal cells are renowned as a powerful biotechnology platform for the production of a wide range of value added products. These include biofuels, animal and aquaculture feeds as well as high value commercial products such as pigments, polysaccharides, bioplastics and organic compounds (Wolf *et al.*, 2015). Microalgae have also been proposed for biorefinery models where multiple compounds can be produced simultaneously from harvested microalgal biomass (Rahman *et al.*, 2015). Microalgae cultivation requires large amounts of water and nutrient supply which consequently turns commercial scale microalgae cultivation to an economically inefficient process (Ebrahimian *et al.*, 2014).

Cultivation of microalgae in wastewater has long been recognized as a viable option for sustainable biomass production and wastewater treatment (Batista *et al.*, 2014, Brennan and Owende, 2010, Pittman *et al.*, 2011, Brown and Shilton, 2014, Rawat *et al.*, 2011, Ruiz-Martinez *et al.*, 2012). The main nutritional requirement for algal growth includes N, P, C and micronutrients such as iron (Fe), magnesium (Mg) and calcium (Ca) which are present in

wastewater. Recent developments in microalgal research have demonstrated that microalgae have the required metabolic potential to effectively reduce high concentrations of nutrients such as C, P and N present in different wastewater streams (Cai *et al.*, 2013). Therefore, microalgae can be used to serve the dual purposes role for the treatment and polishing of the wastewater as well as generating biomass for various applications. Cost reductions by the utilization of wastewater have been reported to be significantly high (reduced from 3 € kg⁻¹ biomass using fertilizers to 1.8 € kg⁻¹ biomass using wastewater) (Acien *et al.*, 2012).

Various wastewater streams including municipal (Lee *et al.*, 2015, Selvaratnam *et al.*, 2014), industrial (Dianursanti *et al.*, 2014, Hernandez *et al.*, 2013, Kamyab *et al.*, 2015, Pathak *et al.*, 2015, Zhou *et al.*, 2014), and agricultural wastewaters (Chen *et al.*, 2015, Cheng *et al.*, 2013, Guldhe *et al.*, 2017, Zhou *et al.*, 2014) as well as primary and secondary effluents, concentrates and anaerobic digestion effluents (Ji *et al.*, 2014, Yang *et al.*, 2015) were exploited as suitable nutrient media for microalgae cultivation. Each wastewater stream has its own characteristics and challenges such as nutrient variability and the presence of potential inhibitors that could impact microalgal growth (Ji *et al.*, 2014). Recently many researchers have developed strategies to overcome the challenges such as low nutrients, high turbidity, bacterial contamination and toxic materials associated with various wastewaters. The types of wastewater utilized for algae cultivation as well as the modes of cultivation (photoautotrophy, mixotrophy and heterotrophy) also affect the scope of biomass for various applications.

2.2. Domestic wastewater composition and treatment process

2.2.1. Domestic wastewater composition

Municipal/Urban wastewaters are a mix of industrial (5-20%) and domestic (80-95%) influents, where the percent composition varies significantly depending on local activities. The typical constituents of municipal/urban wastewater include organic materials, biodegradable COD, nutrients (N and P), metals, inorganic materials and pathogenic microorganisms (Henze and Comeau, 2008). Most municipal wastewaters are rich in nutrients such as ammonia (NH_3), phosphate (PO_4^-), and other essential nutrients for microalgal growth (Mobin and Alam, 2014). Municipal wastewater also contains a variety of inorganic substances from domestic and industrial sources. Low nutrient (N and P) compositions and varying organic loads of municipal wastewater can be limiting factors for microalgal cultivation. Similarly, microorganisms such as bacteria and pathogens that are found in large numbers in municipal wastewater may negatively impact microalgal growth either by direct attack through cell-to-cell contact or indirect attack mediated by extracellular compounds (Wang *et al.*, 2013b). They may also compete with microalgae for essential nutrients (Pittman *et al.*, 2011).

2.2.2. Wastewater treatment process

The most common type of wastewater treatment is activated sludge. The activated sludge process involves the utilization of a consortium of mixed microbial communities through an aerobic biological process to degrade wastewater components (LaPara *et al.*, 2000; Leu *et al.*, 2012). Conventional treatment processes consists of primary, secondary and tertiary treatment phases (Pittman *et al.*, 2011). These phases of treatment involve the physico-chemical or

biological breakdown of complex organic compounds into simpler, more stable compounds in wastewater (Topare *et al.*, 2011). The primary treatment phase encompasses sedimentation of solid materials. Whilst the secondary phase comprises the removal of suspended as well as dissolved organic materials by biological nutrient removal. The final tertiary treatment phase involves the removal of dissolved inorganic compounds that includes nitrogen and phosphorous. Researchers have discovered that microalgae have the potential to be utilized for wastewater treatment due to their substantial nitrogen and phosphorous removal efficiencies (Abdulsada, 2014, Gentili, 2014, Pittman *et al.*, 2011).

2.3. Adverse effects of wastewater discharge

Contamination of groundwater or surface water bodies with wastewater has several adverse impacts on the environment and the aquatic organisms. The wastewater streams are rich in nutrients such as N and P which can cause eutrophication that stimulates algal blooms in receiving water bodies (Topare *et al.*, 2011). Nutrient overloads also cause unwanted pH changes and algal blooms can lead to cyanotoxin production (Christenson and Sims, 2011). Thus the Directive 98/15/EEC establishes a minimum percentage of the reduction of nutrients before water is discharged into the environment (75% COD, 80% P and 70-80% N) (Caporgno *et al.*, 2015). The pathogenic microorganisms from wastewater are hazardous to human health due to the transmission of waterborne diseases. Therefore, adequate wastewater treatment processes are important to avoid the above mentioned problems (Veschetti *et al.*, 2003).

2.4. Microalgal cultivation using domestic wastewater as a nutrient source

Recently many researchers investigated the potential use of municipal wastewater for microalgae cultivation (Li *et al.*, 2011a, Wang and Lan, 2011, Zhou *et al.*, 2012a). Some challenges of using domestic wastewater for microalgal cultivation are 1) toxicity, 2) varying nutrient composition and 3) microbial competition for nutrients that may hamper algal growth.

When cultivating microalgae in any wastewater it is of importance to investigate the physicochemical and biological characteristics of the wastewater in order to determine its suitability for microalgal propagation as well as cultivation strategy. Komolafe *et al.* (2014) states that wastewater characterization also gives insight on the requirements of nutrient supplementation for microalgal growth and also serves to investigate microalgal wastewater treatment during the period of cultivation. Thus it is of importance to investigate the various wastewater streams within the domestic wastewater treatment process for cultivation of microalgae.

2.4.1. Raw influent

Raw sewage could be used as a nutrient source for microalgal cultivation as it is composed of essential nutrients required for microalgal growth such as N in the form of readily bioavailable ammonia or nitrates, and P in the form of orthophosphates (Komolafe *et al.*, 2014). Concentrations of these nutrients however vary in raw sewages depending on the wastewater treatment plant. A study by Wang *et al.* (2010b) observed 33.4 mgL⁻¹ ammonia and 5.66 mgL⁻¹ of orthophosphates in raw wastewater for the cultivation of *Chlorella* sp. with growth rates

of up to 0.412 d^{-1} . Komolafe *et al.* (2014) reported a concentration of 29.12 mgL^{-1} ammonia and 35.4 mgL^{-1} orthophosphates in raw sewage, with the highest biomass productivity attained in both cyanobacteria-dominated ($0.017 \text{ gL}^{-1}\text{d}^{-1}$) and *Desodermus*-dominated ($0.017 \text{ gL}^{-1}\text{d}^{-1}$) mixed cultures. Komolafe *et al.* (2014) also determined the concentration of total faecal coliforms within the raw influent to be $3 \times 10^7 \text{ CFU } 100 \text{ mL}^{-1}$. Microalgae was capable of nutrient removal with simultaneous removal of coliforms of up to 99.8% with decent biomass production.

Wang *et al.* (2013a) studied the nutrient removal efficiency of *Chlorella* sp. in influent wastewater which comprised of 16.3 mgL^{-1} ammonia and 0.64 mgL^{-1} orthophosphates. The influent was diluted into four concentrations (100%, 75%, 50% and 25%) to explore optimum culture conditions. *Chlorella* sp. was able to grow in most culture systems however highest biomass of 0.278 gL^{-1} was obtained at 50% influent wastewater on day 24 of cultivation whilst 100% influent wastewater was observed to have the slowest growth rates. Thus it can be deduced that microalgal growth is dependent on the initial nutrient composition of the wastewater. Raw sewage is generally composed of high concentrations of nutrients, metals, organic compounds and bacteria. Some of these constituents at high concentrations could be toxic for microalgal growth (Morales-Amaral *et al.*, 2015).

2.4.2. Primary effluent

Primary effluent wastewater is obtained after the sedimentation process prior to biological treatment. Primary effluent is rich in nutrients like N and P essential for microalgal growth. Cho *et al.* (2013) used different types of sewage, including primary effluent to cultivate

microalgae. In their study they pre-treated the wastewater using a 1.2 μm size filter to remove particulate matter and microorganisms. Bohutskyi *et al.* (2015) cultivated microalgae in primary effluent with ammonia concentration of 16 mgL^{-1} and orthophosphate concentration of 5.5 mgL^{-1} . Biological characterization in their study showed bacterial load of 10^8 MPN L^{-1} . Organic carbon present in primary effluent ($270 \text{ mgL}^{-1} \text{ COD}$) may efficiently induce other routes of metabolism i.e., mixotrophic and heterotrophic nutritional modes of cultivation as investigated by Zhang *et al.* (2013) and Zhou *et al.* (2011) on autoclaved wastewater.

Cabanelas *et al.* (2013a) reported cultivation of *Chlorella vulgaris* in various wastewaters. Effluent from primary settling tanks composed of 30.6 mgL^{-1} ammonia and 1.7 mgL^{-1} orthophosphates. The COD was reported to be 160 mgL^{-1} . The biomass yields and growth rates observed in their study were $39.28\text{--}87.4 \text{ mgL}^{-1}\text{d}^{-1}$ and $0.11\text{--}0.19 \text{ d}^{-1}$ respectively. The primary effluent was observed to be less suitable for microalgal biomass propagation as opposed to *C. vulgaris* cultivated in centrate and anaerobic digester effluents. This is because primary effluent as a substrate for the cultivation of microalgae has some limiting factors compared to other types of wastewater (centrate, anaerobic digester effluent, secondary or tertiary effluent). Light availability is one of the main limiting factors due to turbidity. Another limiting factor is that it has a higher bacterial load which competes with microalgae for nutrients and organic carbon and ultimately leads to the decline of microalgal growth.

2.4.3. Secondary/tertiary effluent

Secondary wastewater is obtained after the activated sludge treatment process (Bohutskyi *et al.*, 2015). There has been limited research on the use of secondary sewage effluent as it contains lower nutrient levels as opposed to other wastewater streams and synthetic media. Secondary effluent requires a further tertiary treatment process as specified by strict regulation. However, secondary effluent still has considerable amounts of N and P which can be utilized for microalgal growth. Bacterial loads in the secondary effluent however may have adverse effects on microalgal growth (Boonchai and Seo, 2015). The applied value of secondary effluent have been previously reported by Cho *et al.* (2011), Wang *et al.* (2010b) and others for the reduction of nutrient loads with concurrent microalgal biomass production and lipid accumulation.

Cho *et al.* (2011) assessed effluent water discharged from a secondary municipal wastewater treatment plant for mass cultivation of *Chlorella* sp. for biofuel production. Wastewater characterization in their study revealed 19.1 mgL⁻¹ TN, 1.2 mgL⁻¹ TP and 15.3 mgL⁻¹ COD. It appeared that the microorganisms in the effluent wastewater had adversely affected microalgal growth. Pre-treatment of effluent using filtration by 0.2 µm size filters resulted in the highest biomass and lipid productivity of 74 mgL⁻¹d⁻¹ and 22.9 mgL⁻¹d⁻¹ respectively. The filtration with a proper pore size filter (less than 0.45 µm) or UV-B radiation of a proper dose (over 1620 mJ cm⁻²) are proposed as the suitable pre-treatment methods for secondary effluent prior to microalgal cultivation (Cho *et al.*, 2011). These findings led to a deduction that the utilization of secondary effluent can be realized only when bacteria and other undesired microorganisms are significantly reduced or eliminated preceding its use. The low

N concentration in secondary effluent was able to enhance lipid accumulation in microalgae by providing stress conditions (Chinnasamy *et al.*, 2010). Thus cultivation of microalgae in secondary effluent holds great potential for biofuels application.

2.4.4. Anaerobic effluent/digester centrate

The anaerobic sludge slurry is dewatered by filtration and the liquid solution commonly termed 'digester centrate' is recovered while the solid fractions are disposed of (Morales-Amaral *et al.*, 2015). Other than high N and P content, the anaerobic digester centrate contains micronutrients such as potassium, calcium, magnesium, iron, copper and manganese (Osundeko and Pittman, 2014). Anaerobic digester centrate as a sole nutrient source for the production of microalgae has been previously reported by Dong *et al.*, (2014), Ge and Champagne (2015), Li *et al.*, (2011a), Min *et al.*, (2011) and Morales-Amaral *et al.*, (2015).

Min *et al.*, (2011) reported ammonia, nitrate, phosphate and COD concentrations of 113 mgL⁻¹, 0.35 mgL⁻¹, 215 mgL⁻¹ and 3027 mgL⁻¹ respectively, in municipal wastewater anaerobic digester centrate. Morales-Amaral *et al.*, 2015 used centrate from anaerobic digestion as the sole nutrient source for growing fresh water microalgae *Muriellopsis* sp. and *Pseudokirchneriella subcapitata*. Results indicated that with 40–50% of centrate in the culture medium, the productivity values reached up to 1.13 and 1.02 gL⁻¹d⁻¹ for *Muriellopsis* sp. and *P. subcapitata*, respectively. They have also noted a higher nutrient removal up to 90% for N and P both and a significant reduction in COD (lower than 100 mgL⁻¹). It was noted that concentrations above 50% of centrate can be toxic to microalgae due to the substrate toxicity.

Apart from nutrients that support microalgal growth, the centrate also contains compounds that may inhibit microalgal growth if found at high concentrations such as urea, organic acids, phenols and pesticides (Djelal *et al.*, 2014). Thus the optimal centrate concentration of substrate is necessary in order to successfully utilize anaerobic digester centrate wastewater as a nutrient source for microalgal biomass production.

2.4.5. Centrate/activated sludge liquor

Centrate or sludge liquor obtained from dewatering of waste activated sludge (WAS) have higher N and P concentrations than in any other streams produced during the different phases of wastewater treatment process (Wang *et al.*, 2010b). In general, centrate needs to undergo further treatment to avoid environment pollution which results in extra costs for the treatment plants. Using centrate for microalgae cultivation therefore could offer a sustainable solution to reduce the high nutrient content in centrate (Ledda *et al.*, 2015). It can be applied as either a sustainable growth medium or as a supplement nutrient source for secondary effluent as a growth medium for microalgae (Ledda *et al.*, 2015). Wang *et al.* (2010b) investigated the growth of *Chlorella* sp. using the wastewater collected during different phases (raw, primary, secondary and centrate) and have demonstrated that the growth rate of microalgae and nutrient removal efficiencies were proportional to the nutrient concentration of the wastewater selected for its cultivation with the highest growth in centrate followed by raw wastewater. However, the toxic heavy metals and other chemicals that get concentrated along with other nutrients, as well as the bacterial load may have adverse effects on the success of microalgal cultivation.

Li *et al.*, 2011a reported a strain of *Chlorella* sp. that was adapted to municipal wastewater have successfully grown in centrate. Zhou *et al.*, 2011 identified centrate tolerant strains

(*Chlorella* sp., *Heynigia* sp., *Hindakia* sp., *Micractinium* sp., and *Scenedesmus* sp.) for cultivation in centrate wastewater characterized with 91 mgL⁻¹ ammonia nitrogen, 212 mgL⁻¹ orthophosphates and 2324 mgL⁻¹ COD. High growth rates (0.455 - 0.498 d⁻¹) and higher lipid productivities (74.5 - 77.8 mgL⁻¹d⁻¹) were reported for all the strains selected for their study. These observations were promising showing the potential of centrate as a sole media or as a supplementation to tertiary wastewater for microalgal growth.

2.4.6. Waste activated sludge

Amongst municipal wastewater treatment plants, the activated sludge treatment process is the most predominant. The process generally generates significant quantities of waste activated sludge post treatment (Burger and Parker, 2013). Waste activated sludge is the main by-product of this process. Wastewater treatment plants therefore spend tremendous amounts on capital and operational costs for treatment and disposal of sludge (Li *et al.*, 2008a). However conventional disposal techniques are expensive (approximately 40-60% of the total functioning outflow of the plants) due to treatment of sludge before final disposal (Rani *et al.*, 2012). At the end of the wastewater treatment process the sludge is merely dried in drying beds with subsequent disposal by dumping on landfill sites. Sludge is mainly characterized by bacteria, metals and macronutrients such as N and P as well as inorganic C that may negatively impact on the environment. The nutrients from the sludge can be used for microalgal cultivation. Literature outlines some pre-treatment methods that have been employed for sludge disintegration that facilitates the nutrient release from the sludge. (Table 2.1). Some of these include mechanical, thermal, biological and chemical treatments (Li *et al.*, 2008a).

Table 2.1: Biomass productivities, metabolites production and nutrients removal of microalgae grown in various streams of domestic wastewaters from previous studies

Microalgae	Type of wastewater	Pre-treatment	BP* mgL ⁻¹ d ⁻¹	P= productivity in mgL ⁻¹ d ⁻¹			Nutrient removal %		Ref
				L*	PR*	CR*	TN	TP	
Mixed culture dominated by <i>Chlorella</i> sp., <i>Micractinium</i> sp., and <i>Actinastrum</i>	Domestic wastewater influent	Filtration using House paint filters (196 µm) and sparging with CO ₂	~200	C= 4.9-11.3	-	-	99	99	(Woertz <i>et al.</i> , 2009)
<i>Chlorella</i> sp.	Domestic wastewater secondary effluent	Filtration (0.2 µm)	~72	P= 22.9	-	-		86	(Cho <i>et al.</i> , 2011)
<i>Chlorella kessleri</i>	Centrate wastewater	Filtration using filter cloth (Wypall X70, Kimberly-Clark Professional) and autoclaved	~126	-	-	-	61.1-83.9	12.4-92.4	(Li <i>et al.</i> , 2011b)
<i>Chlorella protothecoides</i>	Centrate wastewater	Filtration using filter cloth (Wypall X70, Kimberly-Clark Professional) and autoclaved	~82	-	-	-	84.4-86.7	13.2-82.5	(Li <i>et al.</i> , 2011b)
<i>Chlorella</i> sp.	Municipal anaerobic sludge centrate	Filtration with glass fiber filters (0.45 µm)	~279	-	-	-	-	-	(Lee and Zhang, 2016)
<i>Aurantiochytrium</i>	Municipal sludge liquid product with glucose	Two-step hydrothermal liquefaction and filtration	-	-	-	-	-	-	(Aida <i>et al.</i> , 2016)

*BP- biomass productivity; L- lipid; PR- protein; CR- carbohydrate

2.5. Microalgal nutritional modes of cultivation

One of the inherent evolutionary advantages of microalgae, which is of vital importance, is their flexibility in switching between nutritional modes (photoautotrophy, heterotrophy and mixotrophy), based upon the available substrate and light condition (Venkata Mohan *et al.*, 2014).

2.5.1. Photoautotrophic microalgae cultivation

Conventionally, microalgae are cultivated photoautotrophically as the natural abilities of microalgae are employed. Microalgae have much higher growth rates than other terrestrial plants. Photoautotrophic microalgae require carbon dioxide, inorganic nutrients and light energy to grow and reproduce (Demirbas, 2011, Mutanda *et al.*, 2011). The general interest of employing photosynthetic microalgae for biodiesel production is that they are capable of directly producing oil at the expense of atmospheric CO₂ and sunlight (Li *et al.*, 2008b, Liu *et al.*, 2011).

It has been estimated that more than half of the oxygen on earth is derived from microalgal photosynthesis (Tabatabaei *et al.*, 2011). Photosynthesis takes place in the chloroplast which is controlled by light and dark reactions. Microalgae contain chlorophyll *a* as their primary photosynthetic pigment (Lv *et al.*, 2010). During photoautotrophic growth light is absorbed and water is split by the photosystems to produce oxygen and energy rich compounds such as adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH).

During photosynthetic light reactions, incorporation of one CO₂ to glucose requires three ATP and two NADPH. Thus, these energy rich compounds are used to fix carbon dioxide that is converted into sugars in the Calvin cycle that serve as building blocks for product formation and biomass production. Photosynthetically fixed CO₂ in the form of glucose functions as the sole energy source for all microalgal cellular metabolic activities. (Chang *et al.*, 2011, Kliphuis, 2010, Venkata Mohan *et al.*, 2014).

The photosynthetic dark reactions make use of the Calvin cycle and are comprised of three phases; (1) CO₂ fixation, (2) reduction and (3) regeneration. Carbon dioxide fixation is achieved by the formation of two molecules of 3-phosphoglyceraldehyde (PGA) by the enzyme ribulose biphosphate carboxylase/oxygenase (Rubisco). PGA is then reduced in the reduction phase to glyceraldehyde-3-phosphate (G3P). During the regeneration phase, RuBP is regenerated from G3P and sugars i.e. fructose and glucose are synthesized as energy sources. The production of fructose or glucose from CO₂ requires the Calvin cycle to be operable for at least six times in order to yield the desired hexoses for reformation of the six RuBP molecules. The glucose molecules assist in microalgal lipid biosynthesis under nutrient limitation/stress conditions as a survival/coping mechanism. Commercial scale microalgal cultivation in raceway/open ponds are commonly operable using photoautotrophic mode (Mata *et al.*, 2010, Venkata Mohan *et al.*, 2014).

Microalgae cultivated photoautotrophically have various advantages apart from their excellent capabilities in assimilating nutrients from the environment. However there are drawbacks in the commercialization of biomass production via photoautotrophic mode. This mode requires sufficient illumination for photosynthetic efficiency but suffers low biomass production due to cellular self-shading. Furthermore, low biomass concentrations attained

during photoautotrophic cultivation necessitates the utilization of large, shallow ponds (Cheirsilp and Torpee, 2012, Liu *et al.*, 2011, Perez-Garcia *et al.*, 2011).

2.5.2. Heterotrophic microalgae cultivation

As compared to algae grown photoautotrophically, heterotrophic and mixotrophic algal nutritional modes are known to be much faster with high cellular oil content suitable as a biofuel feedstock (Bhatnagar *et al.*, 2011, Guldhe *et al.*, 2017, Miao and Wu, 2004). As opposed to photoautotrophic microalgal cultivation, heterotrophic cultivation involves growth in the absence of light. In the absence of light with the presence of an organic carbon source, algal photosynthetic processes become suppressed and energy is generated from alternative organic processes that allow for the conversion of sugars into lipids (Perez-Garcia *et al.*, 2010). Miao and Wu (2006) reported that lipid content within heterotrophic microalgae biomass is much higher than in photoautotrophic biomass.

The supplementation of C sources and risk of contamination are the major bottlenecks hindering commercial scale applications of heterotrophic mode of microalgal cultivation (Liang, 2013, Turon *et al.*, 2014). According to a previous estimate by Gao *et al.* (2010), the cost of glucose as the main C substrate accounted for 80% of the total medium cost. Thus investigations using low-cost alternative C sources are required. Waste substances that contain organic C such as wastewater, organic acid end products from fermentations and sugarcane molasses may have potential to replace sugars (Liang, 2013). The use of a wastewater stream for heterotrophic cultivation may be feasible for large scale production of microalgae because

of its organic C load. The reuse of this C from wastewater by microalgae assimilation may greatly reduce cultivation costs of microalgae (Zhang *et al.*, 2013).

2.5.3. Mixotrophic microalgae cultivation

Some algal strains can combine autotrophic photosynthesis as well as heterotrophic assimilation of C in a mixotrophic process (Brennan and Owende, 2010). Organic C and CO₂ are simultaneously assimilated and both respiratory and photosynthetic metabolism function concomitantly (Ebrahimian *et al.*, 2014). Heterotrophic and mixotrophic modes show promise over photoautotrophy in terms of higher growth and lower-cost biomass harvesting (Devi and Mohan, 2012). Thus the cultivation of microalgae by mixotrophy could be a sustainable approach in attaining large amounts of biomass coupled with high growth rates with the additional advantage of photosynthetic metabolite production (Perez-Garcia *et al.*, 2011).

According to literature, studies have observed an increased lipid content in mixotrophic microalgal cultivation as opposed to photoautotrophic and heterotrophic cultivations using synthetic C and nutrient sources (Lin and Wu, 2014). However, a limited number of studies have successfully induced heterotrophic/mixotrophic microalgae production using waste materials as C and nutrient sources. Pleissner *et al.* (2013) heterotrophically cultivated microalgae *Schizochytrium mangrovei* and *Chlorella pyrenoidosa* on fungal hydrolysate from food waste as a nutrient source to produce approximately 20 g of high quality biomass from a 2 L stirred and aerated bioreactor at the end of the fermentation. With regard to mixotrophic algal cultivation, literature available on the use of waste substrates is limited. Studies using alternative cultivation modes to photoautotrophic are scarce due to the high cost implications

of utilizing conventional C sources such as glucose. Thus it is imperative to investigate different microalgal cultivations modes using wastewaters.

2.6. Overview of microalgal applications

Microalgal biomass is comprised of lipids, carbohydrates, proteins and other bioactive compounds. These constituents find their application in various fields such as biofuels, feed, nutraceutical and pharmaceutical. Blue-green algal species i.e. *Nostoc*, *Arthrospira* (Spirulina) and *Aphanizomenon* have been applied as food for thousands of years (Jensen *et al.*, 2001). However, microalgal cultivation has been studied for only a few decades (Borowitzka, 1999). During the early 1950's the world's increasing population and predictions of a short supply of protein sources, led to the examination of unconventional protein sources. Microalgae had come to the forefront of this research as an excellent candidate (Becker, 2007b). In the meantime, the systematic investigation of biologically active substances, pigments, antibiotics in particular, had begun (Borowitzka, 1995). Heightened interest was provoked in the use of microalgae as a renewable energy source during the energy crisis of the 1970's (Chaumont, 1993).

In the early 1960's, large-scale cultivations of microalgae had commenced in Japan with the culture of *Chlorella* by Nihon Chlorella (Borowitzka, 1999, Iwamoto, 2007). This was followed by the establishment of the *Arthrospira* culturing and harvesting facility in Lake Texcoco by Sosa Texcoco S.A. in Mexico during the 1970's (Borowitzka, 1999). Aquaculture fields also first appeared in the 1970's (Pulz and Scheibenbogen, 1998). By the 1980's, 46 large-scale factories were established in Asia. More than 1000 kg of microalgae, predominantly *Chlorella*, was being produced per month. The commercialization of *Dunaliella salina* as a β -

carotene source, had developed into the third major microalgal industry when production facilities were established by Western Biotechnology in Australia and Betatene in 1986. Commercial plants in the USA and Israel soon followed. Large-scale production of blue-green cyanobacteria had emerged in India at about the same time. More recently, the production of *Haemotococcus pluvialis* as a source of Astaxanthin have been achieved in several plants in the USA and India. Thus the microalgal biotechnology industry significantly expanded and diversified in a short period of 30 years. Currently, approximately 5000 t of dry matter/year of microalgae biomass is produced with the generation of a turnover of approximately US\$ 1.25×10^9 /year (processed products excluded) (Pulz and Gross, 2004).

2.6.1. Applications of lipids from microalgae

Microalgal lipids are an appealing feedstocks for biofuels. Employment of microalgae for biodiesel production is advantageous as they are easy to cultivate and have short doubling times. Furthermore, they utilize and convert carbon dioxide to produce oils more efficiently than oil crops (Chisti, 2007). From the standpoint of biodiesel, the more efficient a plant is in the conversion of solar energy into chemical energy, the more superior it becomes. Microalgae are the greatest photosynthetically efficient plants on the globe. Though promising, microalgal biomass for biodiesel production is an expensive process (Chisti, 2007, Demirbas and Demirbas, 2011). In order to lower the cost of producing microalgal oil as biodiesel cheap carbon sources and nutrients should be utilized during the cultivation stage (Huang *et al.*, 2010).

Microalgal lipids have garnered interest primarily from the fact that microalgae are capable of synthesizing considerable amounts of polyunsaturated fatty acids (PUFAs) that have nutritional and pharmaceutical potential (Doughman *et al.*, 2007, Kyle, 2001). Traditional sources of PUFA are from fish which bioaccumulate their PUFAs through the food chain. However, this bioaccumulation depends largely up on the quality and abundance of the fish whereas in algae it does not (Bellou *et al.*, 2014). Ryckebosch *et al.* (2014) evaluated the nutritional value of photoautotrophic microalgal omega-3-long chain PUFAs. The authors discovered that microalgae comprise of ample omega-3-long chain PUFAs to serve as a substitute to fish oil. Microalgal oil consumption ensures the intake of carotenoids which could add nutritional value in comparison to fish oil.

2.6.2. Protein applications of microalgal biomass

Apart from microalgae biofuels, recent feasibility studies have outlined that an integrated biorefinery approach is required for profitable biofuel production (Wijffels *et al.*, 2010, Williams and Laurens, 2010). Proteins are one of the major biochemical constituents found in microalgae and contribute up to 50% of whole cells, thus are expected to play a vital role in the biorefinery approach (Williams and Laurens, 2010). The nutritional quality of microalgal proteins are of interest as they are comparable to food proteins with regard to good profile and proportions of amino acids (Becker, 2007a). Certain microalgal species are known to comprise of comparable protein levels to conventional protein sources such as meat, egg, soybean and milk (Bleakley and Hayes, 2017). However the protein contents of microalgae vary according to the cultivation conditions provided.

Stehfest *et al.* (2005) states that proteins are the main biomass composite of microalgae that are susceptible to reduction when lipids or carbohydrates are accumulated during stress conditions. Conversely, during normal growth conditions and under N supplementation, the protein content increases (Safi *et al.*, 2014). The amino acid profile of the proteins are an indication of their nutritional value (Becker, 1994, Safi *et al.*, 2013). Like most microalgal species, *Chlorella* sp. shows favourable amino acid profile that are enhanced over the standard profile proposed by the Food and Agricultural Organization (FAO) and World Health Organization (WHO) for human nutrition. Thus microalgae proteins open the gate for valorisation options in the food sector market. However research on mass microalgal biomass production is still at the embryonic level (Barka and Blecker, 2016). Thus it is important to investigate microalgal biomass and protein production in waste substrate under different cultivation modes to determine whether they are suitable for application in the food sector.

Apart from human nutrition, lectins and phycobiliproteins are bioactive algal proteins that have medicinal, food and diagnostic applications (Bleakley and Hayes, 2017). Lectins are found to have carbohydrate binding capacity with high specificity. They are applied in blood grouping, anti-viral human immunodeficiency virus type 1 (HIV-1), cancer biomarkers and targets for drug delivery (Aabgeena *et al.*, 2007). Lectins from algae have not been extensively studied as opposed to plant lectins however, their observed bioactivities include anti-viral (HIV-1), mitogenic, cytotoxic, anti-inflammatory, antibacterial, platelet aggregation inhibition and anti-adhesion (Harnedy and FitzGerald, 2011). On the other hand phycobiliproteins are applied in flow cytometry, fluorescent labelling, fluorescent immunohistochemistry and fluorescent microscopy (Aneiros and Garateix, 2004, Kronick and Grossman, 1983). However the conventional application of phycobiliproteins are as natural dyes (particularly phycocyanin used as a blue pigment in chewing gum, confectionary, soft drinks, popsicles, wasabi and dairy

products, cosmetic products such as eyeliner and lipstick) (Spolaore *et al.*, 2006). Patents have also been filed regarding the bioactivities of phycobiliproteins that have health benefits in nutraceuticals such as anti-viral, neuroprotective, hepatoprotective, anti-oxidative, anti-inflammatory and anti-tumour activities (Sekar and Chandramohan, 2008).

Microalgal proteins have further application as animal feed, including farm animals (pigs, poultry and ruminants), pets and aquaculture. It is estimated that approximately 30% of international algal production is used for animal feed, particularly with 50% of *Spirulina* biomass as supplements as a result of its outstanding nutritional profile (Yamaguchi, 1996). *Spirulina* and *Chlorella* are some of the species that are capable of being combined into the diets of pigs, cattle, sheep, poultry and rabbits (Holman and Malau-Aduli, 2013). The most extensive application of microalgal animal feed has been in poultry feed due to its promising prospects for commercial improvement.

Aquaculture feed from algal meal has been gaining increasing interest as it is a rich source of protein, carotenoids, micronutrients and vitamins that can be directly utilized in aqua feeds (Subhadra and Grinson, 2011). This is an attractive advantage as the demand for fish for human consumption has escalated due to open ocean fishing and its related environmental concerns (Anemaet *et al.*, 2010). Microalgae as a dietary supplement have been used to refine aquaculture products to enhance their valorisation. The incorporation of carotenoid pigments from *Haematococcus pluvalis* into the diets of shrimp, lobsters, salmonoids and crayfish have given them their pink flesh characteristic (Muller-Feuga, 2000). In the same respect, microalgal pigments such as Astaxanthin (30 ppm) significantly increases the colour intensity and pattern

of ornamental fish like goldfish, damos, tetras, gouramis, damos, cichlids and koi, enhancing their market value by several fold (Lorenz and Cysewski, 2000).

2.6.3. Carbohydrates from microalgae

Biomass has been noticed as a promising resource for the generation of biofuels such as biodiesel and bioethanol (Ho *et al.*, 2010). At present, the main feedstocks for bioethanol are sucrose and starch crops (sucrose and sugarcane) and lignocellulosic materials (rice and switchgrass) (Nigam and Singh, 2011). However the use of these agricultural crops or wastes are problematic due to escalated demands on water supplies and arable land. The high costs in the conversion process of lignocellulosic material into ethanol also becomes challenging as the high lignin content makes the saccharification process difficult (Sun and Cheng, 2002).

Commodities such as carbohydrates are becoming less available and expensive at a rapid rate. Utilizing microalgae as a sustainable carbohydrate source is a prospect that should be further investigated as these compounds generally represent a large fraction of microalgal biomass (Draaisma *et al.*, 2013). Certain microalgal species are high in carbohydrate contents with starch and cellulosic composites that have potential as feedstock for the production of bioethanol (Dragone *et al.*, 2011). The carbohydrates are also decent candidates for the production of biogas and bio-oils (Chen *et al.*, 2015). However, majority of microalgal strains have high protein content and low carbohydrate contents that might be constraints in utilizing this technology (Markou *et al.*, 2012). Nevertheless, microalgal carbohydrate contents may be manipulated by the conditions provided during cultivation.

2.7. Motivation of study/ research gaps

Microalgal biomass has come to the forefront of applied research for its outstanding potential as fuel and feed commodities in comparison to traditional feedstocks. The process of biomass generation is however too expensive and thus necessitates research into methods of cost reduction. The cultivation of microalgae using chemical media or fertilisers accounts for up to 30% of the total cost of production. The utilisation of nutrient-rich waste substrates have been suggested as alternatives as they are freely available and may provide some environmental benefit such as low-cost wastewater treatment and waste management of sludge. This study focuses on evaluating the suitability of various domestic waste streams and waste solids for microalgal biomass production. Cultivation strategies were developed which comprised of characterization of the waste substrates, various pre-treatments for elimination of undesired microorganisms and nutrient release, and supplementation for maximum biomass production. Biomass generated was analyzed for its biochemical composition (lipids, carbohydrates and proteins).

CHAPTER THREE

ASSESSMENT OF MUNICIPAL WASTEWATERS AT VARIOUS STAGES OF TREATMENT PROCESS AS POTENTIAL GROWTH MEDIA FOR *CHLORELLA SOROKINIANA* UNDER DIFFERENT MODES OF CULTIVATION

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3.1. Introduction

Wastewater contains a combination of organic matter, nutrients and synthetic compounds. It also encompasses majority of the nutrients required for microalgal cultivation and thus can be used for biomass production (Morales-Amaral *et al.*, 2015). Recently different domestic wastewaters have been investigated for microalgal cultivation (Abinandan and Shanthakumar, 2015, Devi and Mohan, 2012, Zhang *et al.*, 2013). However, most of the studies direct their attention to individual wastewaters and photoautotrophic mode of cultivation. Moreover, previous studies focus on either biomass production giving emphasis on lipids or nutrient removal as a treatment process. There are knowledge gaps especially in the identification of a suitable wastewater stream from the domestic wastewater treatment process, investigating different modes of cultivation, pre-treatment methods to minimize the bacterial load, achieving biomass productivities comparable to synthetic media and biochemical composition analysis

of biomass for its suitable application (Abinandan and Shanthakumar, 2015, Pittman *et al.*, 2011, Rawat *et al.*, 2011). The present work focuses on addressing these knowledge gaps.

Species of the *Chlorella* and *Scenedesmus* genera show high tolerance to sewage effluent conditions (Pittman *et al.*, 2011). *Chlorella sorokiniana* was selected for this study for its adaptability towards wastewater and different modes of cultivation (Kim *et al.*, 2013, Ramanna *et al.*, 2014). In this study various waste streams from the different treatment stages of a domestic wastewater treatment plant were selected viz. wastewater influent after primary screening (INF), return activated sludge liquor (RASL), anaerobic tank centrate (AC) wastewater and final effluent (FE) following disinfection by chlorination. Influent is rich in nutrients and its successful use for microalgal cultivation could provide an alternative for the conventional treatment process. Return activated sludge liquor and anaerobic centrate are the waste streams generated during the conventional treatment process which also compose of algae essential nutrients and is generally directed to the head of the treatment plant for further treatment (Rusten and Sahu, 2011). Thus the use of these two streams for microalgal cultivation can reduce the load on the treatment process. Final effluent can be problematic as eutrophication may occur in receiving water bodies due to residual nutrients. Thus the use of final effluent as a microalgal cultivation medium can further remove nutrients for its safe discharge.

The objectives of this aspect of the research were to (1) study the physico-chemical characteristics of waste streams from the different treatment stages of the domestic wastewater treatment plant; (2) to determine the effect of various pre-treatment methods on reduction of bacterial load for efficient algal growth under different cultivation modes; (3) to investigate

nutrient removal from wastewater; (4) nutrient supplementation strategy to achieve high biomass productivities and (5) to biochemically characterize microalgal biomass.

3.2. Materials and methods

3.2.1. Microalgal strain

Microalgal strain *Chlorella sorokiniana*, isolated from the Durban region, KwaZulu-Natal, South Africa, was grown and maintained in sterilized Blue-Green (BG11) medium. Ampicillin $1 \mu\text{g mL}^{-1}$ was added to the medium to minimize susceptibility to bacterial contamination. *Chlorella sorokiniana* was exposed to an irradiance of approximately $120 \mu\text{mol photons m}^{-2}\text{s}^{-1}$, light:dark-cycle of 16:8 h, at room temperature ($25 \pm 1^\circ\text{C}$) with orbital shaking (Labcon, South Africa) at 120 rpm.

3.2.2. Collection and characterization of different wastewater

Domestic wastewater samples (INF, RASL, AC and FE) were obtained in 25 L batches from the Kingsburgh Wastewater Treatment Works in Durban, South Africa ($S30^\circ04.29'$; $E30^\circ51.26'$). Prior to use, wastewater samples were filtered to remove large solids. Samples were then characterized for physico-chemical as well as biological parameters. Temperature and pH were measured using YSI meter (YSI MPS 556: Handheld Multi-Probe Meter, USA). Turbidity was measured using a turbidimeter (2100P-HACH). Concentrations of nitrate ($\text{NO}_3\text{-N}$), nitrite ($\text{NO}_2\text{-N}$), ammonia ($\text{NH}_4\text{-N}$), and phosphate ($\text{PO}_4\text{-P}$) were determined using a

colorimetric method (Thermo Scientific™ Gallery™ Automated Photometric Analyzer, Germany). Chemical oxygen demand (COD) was measured using colorimetric standard methods (Eaton *et al.*, 2005). Quantification of metals were carried out using a modified method by Suthar *et al.* (2009) and analysis was conducted with Agilent 4200 Microwave Plasma-Atomic Emission Spectrometer (MP-AES). Wastewater samples were subjected to biological analysis via heterotrophic plate counts (HPC) on R-2A agar plates using the spread plate method as per standard methods (Eaton *et al.*, 2005).

3.2.3. Pre-treatment of wastewater

Chemical, biological, physical and thermal pre-treatments were applied to wastewaters. Chemical pre-treatments: 5 M NaOH was used to increase the pH of the wastewaters to 11 and 5 M HCl was used to lower the pH to 2 (Xu *et al.*, 2015). Chemicals were added in a dropwise manner and changes in pH were monitored with a pH meter (Thermo Scientific™ Orion™ DUAL STAR™ Meter, UK). For biological pre-treatment, 1 $\mu\text{g mL}^{-1}$ of ampicillin antibiotic was used. Physical pre-treatment involved filtration of the wastewaters using a pressure membrane filtration unit with Whatman 0.45 μm glass microfiber filters (Wang *et al.*, 2010b) (Millipore Corp., Bedford, MA) (Milli Pore, USA) attached with Chemical Duty Pump (WP6122050, 220 V/50 Hz, Millipore, USA) to remove particulates and microorganisms. For thermal pre-treatment, wastewaters were subjected to autoclaving at 121°C for 15 min in accordance with Zhou *et al.* (2012b). Wastewaters were biologically characterized before and after pre-treatments in order to determine the most effective and feasible pre-treatment method in reducing the microbial load of the wastewaters for microalgal cultivation.

3.2.4. Cultivation of microalgae

3.2.4.1. Microalgal cultures in synthetic media

Chlorella sorokiniana was cultivated in BG11 medium. The addition of ampicillin (1 $\mu\text{g mL}^{-1}$) was used in all synthetic media to prevent bacterial contamination. The cultivation experiments were performed using 500 mL BG11 medium with the pH adjusted to 7.0 at the time of inoculation in 1 L flasks. The cultures were subjected to incubation at room temperature and orbital shaking at 120 rpm. For photoautotrophic cultivation, cultures were illuminated at approximately 120 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ with a light:dark-cycle of 16:8 hr. For mixotrophic and heterotrophic cultivations, glucose was used as a carbon source at a concentration of 5 g L^{-1} with and without illumination, respectively (Kim *et al.*, 2013).

3.2.4.2. Microalgal cultures in wastewater

Post pre-treatment of wastewaters involved the inoculation of *C. sorokiniana* into 500 mL INF and AC in 1 L Erlenmeyer flasks. The cultures were incubated at room temperature and subjected to orbital shaking at 120 rpm. Mixotrophic cultures were illuminated at approximately 120 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ with a light:dark-cycle of 16:8 h whilst heterotrophic cultures were light deprived by covering Erlenmeyer flasks with aluminum foil and kept in dark conditions. Mixotrophic and heterotrophic experimentation was carried out in duplicate over a period of 14 and 8 days respectively.

3.2.4.3. Nitrogen supplementation in wastewater for microalgal cultivation

To enhance biomass productivity, urea (500 mgL⁻¹ and 1500 mgL⁻¹) was supplied as a nitrogen source into AC for the subsequent growth studies of *C. sorokiniana*. The experiment was carried out under mixotrophic mode of cultivation for 14 days as these were observed to be the optimal waste substrate and mode in terms of biomass productivity. Modified BG11 medium (with the addition of 5 gL⁻¹ glucose) and filtered AC with no addition of urea were used as the positive and negative controls respectively. Concentrations of NO₃-N, NH₄-N, PO₄-P and (COD) were determined pre and post cultivation as described in section 3.2.2.

3.2.5. Analytical methods

Optical density (OD₆₈₀) readings were taken daily using a spectrophotometer (SpectroquantRPharo 300, Merck, Germany) to monitor microalgal growth. Biomass concentrations expressed in g l⁻¹ were determined using dry cell weight (DCW) measurements by gravimetric analysis. Dry cell weight was determined once every 2 days. Chlorophyll *a* and *b* contents were determined once every 2 days in accordance with Pancha *et al.* (2014). Chlorophyll content was calculated according to the following equations:

$$\text{Chlorophyll } a; \text{ Chl-}a \text{ (}\mu\text{g mL}^{-1}\text{)} = 16.72 A_{665.2} - 9.16 A_{652.4} \quad (1)$$

$$\text{Chlorophyll } b; \text{ Chl-}b \text{ (}\mu\text{g mL}^{-1}\text{)} = 34.09 A_{652.4} - 15.28 A_{665.2} \quad (2)$$

Pulse Amplitude Modulation (PAM) fluorescence was used to determine the photosynthetic performance of microalgae when subjected to cultivation in synthetic media

and wastewaters under different cultivation modes. Fluorescence measurements were obtained non-invasively using a Dual-PAM 100 chlorophyll fluorometer (Heinz Walz GmbH, Effeltrich, Germany). Microalgal cultures were adapted to dark conditions for approximately 20 minutes as Photosystem II (PS II) reaction centers are open and require to be closed before analysis. Rapid photosynthetic light curves were then generated by providing a sequence of increasing actinic irradiance in 15 discrete increments using Dual PAM software v1.9. Cultures were exposed to a Saturation Pulse (SP) for 10 s of actinic light, before a blue light, (0.6 s at 10,000 mol photons m⁻² s⁻¹) was applied to determine the Electron Transport Rate (ETR) for each irradiance level. Photon irradiance (400 to 700 nm) incident on the sample surface were continuously measured using a PAR micro-sensor (Spherical MicroQuantum Sensor US-SqS/W, Waltz) connected to the control unit. Relative electron transport rates (rETR) were recorded as per Ramanna *et al.* (2014).

$$rETR = F'_q / F'_m \times PPF D \quad (3)$$

where $F'_q = (F'_m - F')$, F'_m is maximum fluorescence in a light adapted sample and F' is dark fluorescence yield. F'_q / F'_m is the Photosystem II (PS II) operating efficiency and provides estimations of the efficiency of light usage absorbed by PS II. This parameter provides an estimate of the quantum yield of linear electron flux through PS II at a particular photosynthetically active photon flux density (PPFD).

Maximum quantum efficiency of photosystem II charge separation (F_v/F_m) was calculated in accordance with Ramanna *et al.* (2014):

$$F_v/F_m = F_m - F_o / F_m \quad (4)$$

where F_m is the maximum fluorescence in a dark adapted sample and F_o is the minimum fluorescence in a dark adapted sample, which results in the variable fluorescence F_v .

Lipid extraction from dry biomass was carried out using solvent mixture methanol:chloroform (2:1) assisted with microwave cell disruption (Milestone S.R.L., Italy, output power 1200 W) using at 100°C for 10 min at 1000 W (Guldhe *et al.*, 2014). The mixture was filtered to separate solvent and cell debris and the filtrate was oven dried at 60°C. Crude lipid was quantified gravimetrically and the lipid yield (%) was calculated.

Protein content was determined using the Bradford assay. Bovine serum albumin (BSA) was used as the protein standard to generate a calibration curve. Microalgal biomass samples were prepared according to methods by (Guldhe *et al.*, 2015) with the addition of 100 µL 1 M NaOH to 10 mg biomass. The mixtures were incubated at 80°C for 10 min and 900 µL of distilled water was added. Mixture was subjected to centrifugation at 12,000 g for 10 min and the supernatants were utilized for the protein assay. Absorbance readings were determined at 595 nm (SpectroquantRPharo 300, Merck, Germany).

Carbohydrate content was assessed colorimetrically using the phenol–sulphuric acid method. Glucose was used as a carbohydrate standard for the generation of a calibration curve. Light absorbance readings were measured at 490 nm (SpectroquantRPharo 300, Merck, Germany) to determine carbohydrate concentrations of microalgae samples (Albalasmeh *et al.*, 2013).

3.2.6. Statistical analysis

Statistical analyses of data were carried out by using Minitab statistical software. Significance of results and differences among various treatments were evaluated for duplicate set of data by using one-way analysis of variance (ANOVA). Posthoc Tukey's test ($P=0.05$) was used for comparisons among the different means. Data was represented as Mean \pm SD (Standard deviation) in tables, while in graphs; SD values were represented as error bars.

3.3. Results and discussion

3.3.1. Screening of wastewaters

Physico-chemical and biological characteristics of each wastewater stream in their native state are reported in table 3.1. The concentrations of $\text{NH}_4\text{-N}$, $\text{NO}_3\text{-N}$, $\text{NO}_2\text{-N}$ and COD were found to be highest in INF followed by AC. The phosphate concentration was found to be highest in AC (24 mgL^{-1}) followed by INF (19.7 mgL^{-1}). The concentrations of $\text{NH}_4\text{-N}$, COD and $\text{PO}_4\text{-P}$ were comparatively lower in RASL (4.24 , 29.28 and 3.68 mgL^{-1} respectively) and FE (4.23 , 86.52 and 0.55 mgL^{-1} respectively). Thus INF with 50.49 mgL^{-1} $\text{NH}_4\text{-N}$, 26 mgL^{-1} $\text{NO}_3\text{-N}$, 474.36 mgL^{-1} COD and 19.7 mgL^{-1} $\text{PO}_4\text{-P}$ and AC with 35 mgL^{-1} $\text{NH}_4\text{-N}$, 10.7 mgL^{-1} $\text{NO}_3\text{-N}$, 185.53 mgL^{-1} COD and 24 mgL^{-1} $\text{PO}_4\text{-P}$ could provide sufficient nutrients for microalgal growth as compared to the other screened wastewater streams collected from different stages of the wastewater treatment process.

Table 3.1: Physico-chemical and biological characterization of wastewaters from different stages of the wastewater treatment process

	INF	RASL	AC	FE
pH	7.48±0.23	6.69±0.33	7.21±0.06	7.07±0.01
Temperature (°C)	21.85±3.24	22.37±2.88	22.06±2.44	23.39±1.18
COD (mgL ⁻¹)	474.36±0.001	29.28±0.001	185.53±0.001	86.52±0.001
NH ₄ -N (mgL ⁻¹)	50.49±0.21	4.24±0.14	35.00±1.27	4.23±0.10
NO ₃ -N (mgL ⁻¹)	26.00±0.001	0.20±0.001	10.70±0.001	0.05±0.001
NO ₂ -N (mgL ⁻¹)	120.00±0.001	0.35±0.001	70.00±0.001	0.02±0.001
PO ₄ -P (mgL ⁻¹)	19.7±0.42	3.68±0.10	24.00±0.001	0.55±0.001
SO ₄ ²⁻ (mgL ⁻¹)	57.27±1.141	76.36±0.381	48.48±3.161	71.76±0.001
Cu ²⁺ (mgL ⁻¹)	14.55±2.16	9.44±0.07	1.08±1.29	0.68±1.13
Co ²⁺ (mgL ⁻¹)	1.33±0.02	1.15±0.09	0.51±0.17	0.51±0.10
Fe ³⁺ (mgL ⁻¹)	64.84±1.93	49.93±1.82	16.35±1.16	21.94±0.59
Mo ⁶⁺ (mgL ⁻¹)	3.24±0.13	0.7±0.10	0.39±0.01	0.17±0.03
Mn ²⁺ (mgL ⁻¹)	1.35±0.08	1.13±0.06	0.79±0.12	0.52±0.10
K ⁺ (mgL ⁻¹)	160.87±7.38	157.49±6.02	168.54±2.30	88.69±0.48
Mg ²⁺ (mgL ⁻¹)	138.87±5.12	108.54±7.98	84.46±1.85	45.31±0.25
Zn ²⁺ (mgL ⁻¹)	145.89±6.51	121.68±3.78	63.14±1.46	30.94±0.46
Na ⁺ (mgL ⁻¹)	717.94±27.35	591.42±4.45	476.63±9.46	389.14±17.38
TSS (gL ⁻¹)	0.06±0.02	0.014±0.04	0.02±0.02	0.01±0.22
TDS (gL ⁻¹)	0.53±0.01	0.4±0.14	0.52±0.03	0.41±0.01
TS (gL ⁻¹)	0.6±0.13	0.41±0.01	0.54±0.20	0.42±0.21
Turbidity (NTU)	23.32±0.07	3.36±0.25	4.15±0.39	2.55±0.13
HPC (CFU mL ⁻¹)	1.92 × 10 ⁷	1.1 × 10 ⁹	2.88 × 10 ⁷	5.4 × 10 ²

Gupta *et al.* (2016b) cultivated *C. sorokiniana* in raw sewage with a composition of 52.23 mgL⁻¹ NH₄-N, 8.47 mgL⁻¹ PO₄-P and 320 mgL⁻¹ COD and observed 86.93% N removal, 68.24% P removal and 69.38% COD removal. A study by Ebrahimian *et al.* (2014) successfully cultivated *Chlorella vulgaris* in primary wastewater comprising of 43.31 mgL⁻¹ NH₄-N, 56.19

mgL⁻¹ NO₃-N, 0.63 mgL⁻¹ PO₄-P and 256 mgL⁻¹ COD. *C. vulgaris* was also grown successfully in secondary wastewater with the following characteristics: 0.63 mgL⁻¹ NH₄-N, 224.78 mgL⁻¹ NO₃-N, 0.53 mgL⁻¹ PO₄-P and 96 mgL⁻¹ COD. Moreover, trace metals such as Cu²⁺, Mn²⁺, K⁺, Mg²⁺ and Fe³⁺ essential for various physiological functions of microalgae were also detected in the wastewaters selected in the present study. In a study by Singh *et al.* (2016a) it was observed that the optimum iron concentration was 9 mgL⁻¹ and optimum magnesium concentration was 100 mgL⁻¹ to obtain high biomass and lipid productivities in *A. obliquus*. Microalgal nutrient requirement and uptake is highly strain specific, this however depends upon the cultivation conditions and initial nutrient concentrations available in the growth medium.

The high COD observed in INF (473.36 mgL⁻¹) and AC (185.53 mgL⁻¹) could provide a carbon source for microalgal mixotrophic and heterotrophic modes of cultivation. Microalgae can utilize these nutrients present in the selected wastewaters and could serve a dual role in nutrient removal as well as biomass production for energy and commodities. The bacterial load was found to be highest in RASL followed by AC and INF respectively. The bacterial load was minimal in FE in comparison to other wastewater streams. Based on nutrient composition, INF and AC were found to be suitable for microalgal cultivation, however the bacterial load needs to be minimized by pre-treatment to eliminate the possible inhibition of microbial growth due to competition for nutrients.

3.3.2. Effect of pre-treatment methods

Microbial load observed in raw INF was 1.92×10^7 CFU mL⁻¹ while that in AC was 2.88×10^7 CFU mL⁻¹. One of the constraints in introducing any wastewater source for cultivating

microalgae is the presence of other microorganisms which may include pathogenic bacteria and predatory zooplankton (Osundeko and Pittman, 2014). These microorganisms compete with algae for nutrients and oxidizable carbon within the wastewater and thus may outcompete the desired microalgal strain. Thermal, physical, biological and chemical pre-treatment methods were therefore applied to the selected INF and AC in order to minimize or eliminate the presence of microorganisms before the cultivation of *C. sorokiniana*.

As seen in table 3.2, thermal pre-treatment (autoclaving) was observed as the most effective pre-treatment method in neutralizing all microbes. Acidification (pH 2) treatment was found to be the second most effective pre-treatment with CFU mL⁻¹ of less than 100 in both the selected wastewaters. Filtration pre-treatment resulted in a microbial load of 6.4×10^4 CFU mL⁻¹ in INF and 8.4×10^4 CFU mL⁻¹ in AC. However, it was observed that autoclaving hampered the nutrient content, significantly reducing essential nutrients required by microalgae. Percentage reductions of ammonia and phosphates were 44.73% and 6.63% respectively after autoclaving treatment. Sriram and Seenivasan (2012) reported that sterilization of wastewater by autoclaving, reduces nutrients and might have an effect on microalgal growth. In such case chemical nutrients are added for growth of microalgae. However, this adds to excessive cultivation costs that become impractical at large scale. Several other wastewater characteristics were altered post autoclaving which were perhaps correlated and possibly may or may not be conducive for microalgal growth. During autoclaving a pH increase was generated (Table 3.2) due to the loss of CO₂, sequentially causing a shift in the CO₂ buffer system and hence the precipitation of salts (essential trace elements and toxic heavy metals). Furthermore, chelating abilities for trace elements and heavy metals of certain compounds might be altered due to transformation post autoclaving (Ho *et al.*, 2013). Also,

appreciable quantities of ammonia were lost (44.73%) from the wastewater through volatilization during autoclaving.

Table 3.2: Effect of thermal, physical, biological and chemical pre-treatments on wastewater bacterial load

Wastewater	Parameter	Raw	Autoclaving	Filtration	Antibiotic	pH 2	pH 11
INF	pH	7.48	9.67	8.14	7.73	2.00	11.02
	HPC (CFU mL ⁻¹)	1.92×10^7	-	6.4×10^4	5.2×10^5	-	1.46×10^6
AC	pH	7.21	9.09	7.86	7.56	1.94	10.88
	HPC (CFU mL ⁻¹)	2.88×10^7	-	8.4×10^4	5.2×10^6	>100	1.01×10^5

Antibiotic treatment resulted in a microbial load of 5.2×10^5 CFU mL⁻¹ in INF and 5.2×10^6 CFU mL⁻¹ in AC. An antibiotic may not have been an effective bactericide for the pre-treatment of wastewaters due to the antibiotic spectrum of activity. Chemical pre-treatment (pH 2) applied in this study was able to significantly reduce the microbial load. However, the acidity of the wastewater post pre-treatment would be deleterious to the growth of microalgae. The added cost of chemicals to neutralize the wastewater for microalgal cultivation, makes this method less appealing for application at commercial scale.

In this study, filtration was observed as the most feasible pre-treatment method over the other applied pre-treatments as it significantly reduced the bacterial load while maintaining the nutrient availability from the original, raw waste substrates. Energy consumption for filtration was found to be 0.07 kWh, while for autoclaving it was 0.62 kWh. Energy consumption of autoclaving was found to be 10 times more to that of filtration. Thus, filtration was selected as

the pre-treatment method for further microalgal cultivation studies in INF and AC under mixotrophic and heterotrophic modes.

3.3.3. Effect of different wastewater and mode of nutrition on the biomass and photosynthetic performance of microalgae

The performance of *C. sorokiniana* was studied to determine its robustness and growth yields in the selected wastewaters INF and AC as growth media under different cultivation modes. The microalgae successfully grew in both wastewaters under mixotrophic and heterotrophic conditions, which implies that these waste substrates are able to support microalgal growth requirements and that *C. sorokiniana* adapted to them despite the presence of bacteria. Figure 3.1 depicts the biomass productivity variation for the microalgal cultivation in different substrates and modes. The obtained biomass productivities were 83.2 ± 29.85 , 201.43 ± 1.01 , 72.5 ± 0.51 , 77.14 ± 3.03 , 173.81 ± 0.09 , 61.88 ± 4.42 and 76.25 ± 3.54 $\text{mgL}^{-1}\text{d}^{-1}$ for cultivations in autotrophic BG11 (Auto BG11), mixotrophic BG11 (Mixo BG11), mixotrophic INF (Mixo INF), mixotrophic AC (Mixo AC), heterotrophic BG11 (Hetero BG11), heterotrophic INF (Hetero INF) and heterotrophic AC (Hetero AC) respectively.

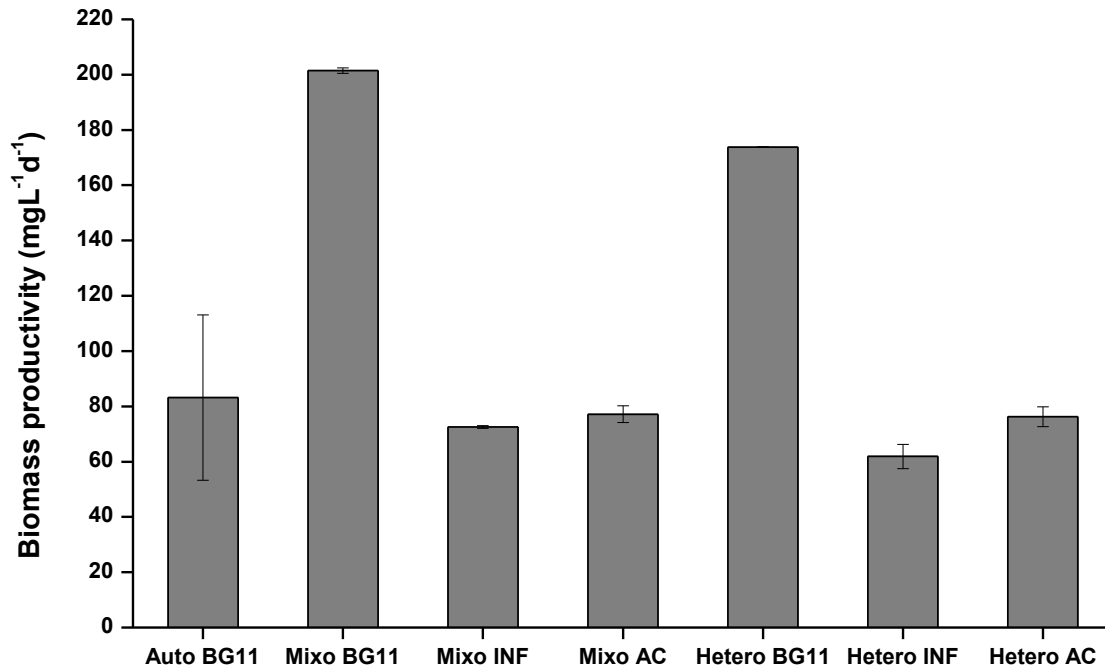


Figure 3.1: Biomass productivities of *Chlorella sorokiniana* in influent and anaerobic centrate under mixotrophic and heterotrophic cultivation modes. Data represented as mean \pm SD (n=2).

Amongst the INF and AC cultivations in different modes, the highest biomass productivity of $77.14 \pm 3.03 \text{ mgL}^{-1}\text{d}^{-1}$ was observed in Mixo AC. Due to the availability of abundant COD and light exposure, mixotrophic cultivations in wastewaters has shown higher biomass productivities compared to heterotrophic mode. The highest biomass productivity ($77.14 \pm 3.03 \text{ mgL}^{-1}\text{d}^{-1}$) coupled with high chlorophyll content and final biomass concentration ($1080 \pm 42.43 \text{ mg l}^{-1}$) was observed in Mixo AC after a 14 day cultivation period. Heterotrophic anaerobic tank centrate culture showed biomass productivity of $76.25 \pm 3.54 \text{ mgL}^{-1}\text{d}^{-1}$. Maintaining dark conditions and competing bacterial growth are the major challenges for heterotrophic mode

specifically at large scale and open systems. Although biomass concentration in Mixo AC was high and relative to microalgal biomass production in Auto BG11 ($1165 \pm 417.9 \text{ mgL}^{-1}$), it was significantly lower in comparison to the microalgal biomass concentration attained from Mixo BG11 ($2820 \pm 14.14 \text{ mgL}^{-1}$). Thus further strategy of addition of an exogenous nutrient source to enhance biomass productivity was employed and investigated for Mixo AC microalgal cultivation.

The rETR is a determination of the rate of linear electron transport through PS II which indicates the photosynthetic performance of microalgae (Singh *et al.*, 2016a). At late log phase, highest rETR value (35.05 ± 0.07) was observed for microalgae culture grown under Auto BG11 cultivation condition followed by culture grown in Mixo AC (33.4 ± 1.5). However, rETR values for Mixo BG11 was low as compared to Auto BG 11 (Table 3.3). This may be due to the presence of glucose being able to exert an opposing influence on photosynthesis as it reduces the fixation of CO_2 and inhibits synthesis of Rubisco-enzymes. Similarly, Liu *et al.* (2009) have reported reduction in rETR values of microalgae *Phaeodactylum tricornutum* grown under mixotrophic conditions using different organic carbon sources. Generally, microalgal cultures having Fv/Fm ratios of above ≥ 0.5 indicates acceptable physiological condition of the culture (Ramanna *et al.*, 2014). In this study at late log phase Fv/Fm ratio for all culture conditions were between 0.53-0.69 (Table 3.3), which indicates decent adaptability of *C. sorokiniana* in different culture conditions.

Table 3.3: Biomass, biomass productivity, chlorophyll (*a* and *b*) content and photosynthetic performance (Fv/Fm) of *Chlorella sorokiniana* cultivated in influent, anaerobic centrate and urea supplemented anaerobic centrate under different cultivation modes

Mode and Substrate	Biomass (mgL ⁻¹)	Biomass Productivity (mgL ⁻¹ d ⁻¹)	Chlorophyll <i>a</i> (µg mL ⁻¹)	Chlorophyll <i>b</i> (µg mL ⁻¹)	Fv/Fm late log	rETR late log
Auto BG11	1165±417.90	83.2±29.85	7.73±0.73	3.78±0.68	0.53±0.02	35.05±0.07
Mixo BG11	2820±14.14	201.43±1.01	10.92±0.08	4.02±0.001	0.69±0.05	22.6±0.28
Mixo INF	1015±7.07	72.5±0.51	2.96±0.27	1.18±0.5	0.64±0.01	23.8±0.001
Mixo AC	1080±42.43	77.14±3.03	3.06±0.72	1.65±0.3	0.61±0.03	33.4±1.50
Mixo AC 500	1144.5±21.92	81.75±1.57	4.63±0.001	0.21±0.1	0.63±0.003	34.8±0.001
Mixo AC 1500	2275±106.07	162.50±7.58	7.37±0.001	0.54±0.001	0.56±0.01	33.6±0.001
Hetero BG11	1390±0.71	173.81±0.09	10.97±0.74	3.78±0.66	-	-
Hetero INF	495±35.36	61.88±4.42	0.08±0.001	0.03±0.001	-	-
Hetero AC	610±28.28	76.25±3.54	0.08±0.02	0.04±0.03	-	-

3.3.4. Microalgal nutrient and organic carbon removal

Nutrient removal efficiencies are relative to the nutrient levels in wastewater and the extent of the utilization of these nutrients by microalgae. Nutrient removal efficiencies of *C. sorokiniana* in Mixo INF, Mixo AC, Hetero INF and Hetero AC are depicted in Table 3.4.

Table 3.4: *Chlorella sorokiniana* nutrient and chemical oxygen demand removal efficiencies in influent and anaerobic centrate under mixotrophic and heterotrophic modes of cultivation

Mode and Substrate	NO₃-N (%)	NH₄-N (%)	NO₂-N (%)	PO₄-P (%)	COD Removal (%)
Mixo INF	19.23	89.13	75.00	87.82	36.68
Mixo AC	28.67	94.29	57.14	83.3	44.03
Hetero INF	56.25	27.91	33.33	13.27	59.15
Hetero AC	25.00	23.53	25.00	83.81	59.42

Microalgae were able to effectively remove NH₄-N in Mixo INF and Mixo AC at rates of 89.13% and 94.29%. However heterotrophic conditions were only capable of allowing removal of 27.91% and 23.53% NH₄-N in Hetero INF and Hetero AC respectively. Microalgal NH₄-N removal is triggered by the direct consumption of NH₄-N and NH₃ stripping. This stripping of NH₃ only occurs under increased temperatures and alkaline conditions. The ammonia is volatilized at high temperatures and pH. Since temperature conditions were kept constant at 25°C, it is possible that the stripping process was insignificant during the cultivations and thus is an indication that microalgal absorption was primarily responsible for the decrease in NH₄-N (Zhou *et al.*, 2012b). Moreover, NH₄-N tolerance is a strain specific response (Morales-Amaral *et al.*, 2015) thus from the results of this study, *C. Sorokiniana* evidently has pronounced tolerance to NH₄-N concentrations particularly under mixotrophic conditions.

Removal rates of NO₃-N were much lower in Mixo INF (19.23%), Mixo AC (28.67%) and Hetero AC (25%) than Hetero INF (56.23%). Literature states that NH₄-N and NO₃-N are

primary nitrogen sources for plants and that both sources can be utilized effectively by *Chlorella* sp. (Bloom *et al.*, 1992). In microalgal cells, assimilation of nitrogen in its oxidized form ($\text{NO}_3\text{-N}$ and $\text{NO}_2\text{-N}$) and reduced form ($\text{NH}_4\text{-N}$) is done via different metabolic processes. Nitrate reductase and nicotinamide adenine dinucleotide (NADH) are responsible for the reduction of $\text{NO}_3\text{-N}$ to $\text{NO}_2\text{-N}$ by the transfer of two electrons during the reaction. Thereafter, nitrite reductase and ferredoxin (Fd) reduce $\text{NO}_2\text{-N}$ to $\text{NH}_4\text{-N}$ by the transfer of six electrons in total during the reaction. Ultimately, all inorganic nitrogen is reduced to ammonium before amino acid incorporation within intracellular fluid. Thus it can be suggested that *C. sorokiniana* preferred $\text{NH}_4\text{-N}$ rather than $\text{NO}_3\text{-N}$ for growth in Mixo AC and Hetero AC as redox reactions are not required in the assimilation of $\text{NH}_4\text{-N}$ and thus requires lesser energy utilization (Ge and Champagne, 2015).

A substantial $\text{PO}_4\text{-P}$ removal rate was achieved by microalgae in Mixo INF, Mixo AC and Hetero AC of 87.82%, 83.3% and 83.81% respectively. This complies with a study by Akerstrom *et al.* (2014) where $\text{PO}_4\text{-P}$ removal efficiencies by microalgae biosynthesis in anaerobically digested sludge liquor were 65-95%. Thus it is evident that *C. sorokiniana* has a high $\text{PO}_4\text{-P}$ uptake capacity as a considerable amount of $\text{PO}_4\text{-P}$ is capable of being adsorbed into microalgal cell walls and stored as reserves. This attribute could be beneficial for prolonged cultivation periods with assimilation of phosphorous after phosphorous depletion in the medium. Microalgae utilize $\text{PO}_4\text{-P}$ as the preferred phosphorous species which resulted in its rapid uptake in this investigation. Phosphorous removal by *C. sorokiniana* was found to be higher than previous studies where *Chlorella* sp. was cultivated in anaerobic digestate dairy manure achieving a removal rate of between 63% and 75% (Wang *et al.*, 2010a) and removal of phosphorous in domestic wastewater by *Chlorella vulgaris* was 80% (Ruiz-Marín *et al.*, 2010).

In this study, *C. sorokiniana* was able to remove 59.15% and 59.42% COD in Hetero INF and Hetero AC respectively which was a higher removal rate as compared to the INF and AC grown mixotrophically, suggesting that microalgae assimilate higher amounts of COD where there is no illumination and can utilize different organic carbon sources other than CO₂ (Wang *et al.*, 2010a, Zhou *et al.*, 2011, Zhou *et al.*, 2012a). Confirmation of this theory was observed in a study by Kim *et al.* (2013) where *C. sorokiniana* was cultivated and achieved COD removal rates of 70% and 65% under heterotrophic and mixotrophic modes respectively.

3.3.5. Nitrogen supplementation to wastewater

Nitrogen (N) is essential for microalgal cultivation and is available in several forms. The form of N supplied to microalgae has various effects on their biochemical composition, growth rates and culture stability (Borowitzka and Moheimani, 2013). Previous studies have utilized urea as the preferred N source to enhance microalgal biomass production from a selection of N sources such as sodium nitrate, ammonium carbonate and ammonium chloride (Khalili *et al.*, 2015, Ramanna *et al.*, 2014). Moreover, from the nutrient removal efficiencies in this study, *C. sorokiniana* preferred ammonium as N source rather than nitrate which was evident by high ammonium removal rates of up to 94.29% as compared to low nitrate removal efficiencies of up to 28.67%. Thus to improve biomass productivity of Mixo AC, urea as an N source was provided as opposed to sodium nitrate. Furthermore, a study by Khalili *et al.* (2015) states that *Chlorella* sp. had high biomass concentration and lipid accumulation when urea was used as a N source. Biomass productivities of *C. sorokiniana* in Mixo AC 500 and Mixo AC 1500 are illustrated in Figure 3.2.

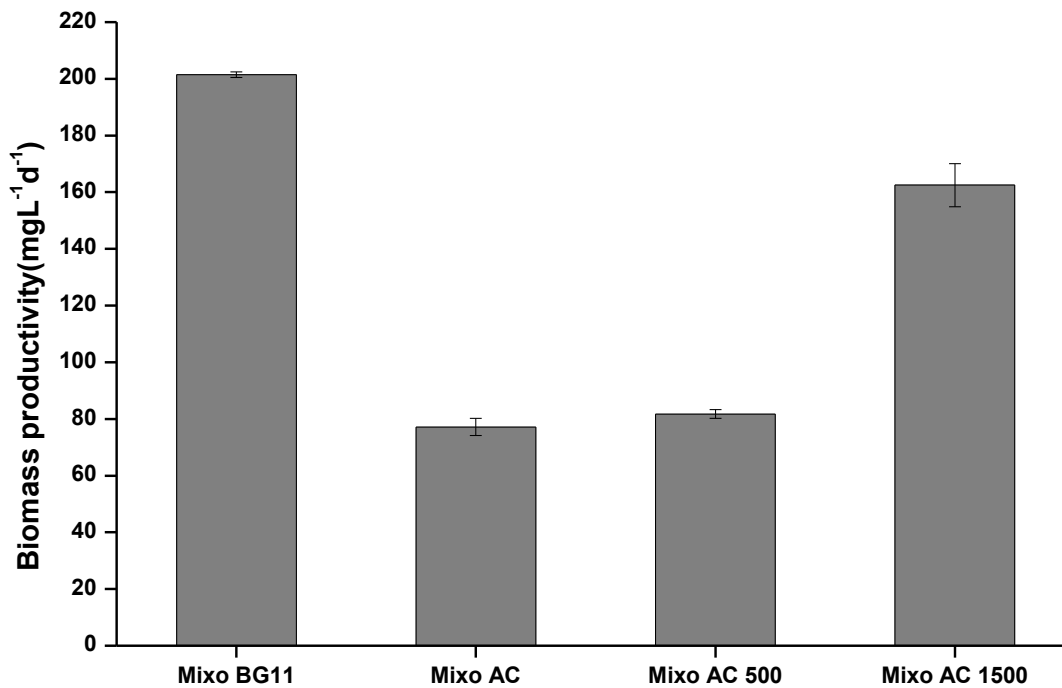


Figure 3.2: Biomass productivities of *Chlorella sorokiniana* cultivated in mixotrophic anaerobic centrate supplemented with urea (500 mgL⁻¹ and 1500 mgL⁻¹). Data represented as mean \pm SD (n=2).

As shown in Table 3.3, biomass productivity in Mixo AC 1500 (162.50 mgL⁻¹d⁻¹) was higher than in Mixo AC 500 (81.75 mgL⁻¹d⁻¹) and in Mixo AC without supplementation (77.14 mgL⁻¹d⁻¹). The biomass productivity achieved in Mixo AC 1500 was around 80% to that achieved in Mixo BG11. These results are promising for developing an inexpensive cultivation strategy, considering a waste substrate was used as the growth medium and a cheap nutrient source was used for supplementation. Moreover, after a 14 day cultivation period the highest microalgal biomass concentrations were found in Mixo BG11 (2820 mgL⁻¹) followed by 2275 mgL⁻¹ in Mixo AC 1500. The finding of high biomass concentration in Mixo AC 1500 is

supported by high chlorophyll content ($7.37 \mu\text{g mL}^{-1}$) and high rETR (33.6) values (Table 3.3). Thus it appears that microalgae were able to dominate over bacteria and other contaminants in the AC. Therefore, it can be concluded that urea can be effectively used as a nitrogen source for enhancing microalgal biomass yields in domestic wastewater.

3.3.6. Biochemical composition analysis

Microalgae biomass contains three major components: lipids, proteins and carbohydrates. Biochemical analysis was done to observe the changes in microalgal biomass in terms of these components when cultivated in various substrates and under different modes of cultivation. These results are represented in Table 3.5. The percentage of the main biochemical components varies depending on the strain of microalgae, the culture medium and the cultivation conditions provided during the cultivation period. During the 14-day photoautotrophic cultivation in BG11, *C. sorokiniana* was able to accumulate a lipid content of 12.70%, protein content of 20.89% and a carbohydrate content of 23.24% as seen in Table 3.5. A study by Wan *et al.* (2012) attained biochemical composition results of 19% lipid content, 13% protein content and 6% carbohydrate content from 30-day photoautotrophically cultivated *C. sorokiniana*. In this study, lipid, protein and carbohydrate composition attained from mixotrophic (15.2%, 23.15% and 36.96% respectively) and heterotrophic (23.7%, 23.66% and 44.50% respectively) cultivated microalgae in BG11, results from Auto cultivations are relatively low. Thus for biomass propagation with increased biochemical components, Mixo and Hetero cultivation modes were investigated for *C. Sorokiniana* in wastewater.

Table 3.5: Biochemical composition of *Chlorella sorokiniana* in BG11 medium, influent, anaerobic centrate and urea supplemented anaerobic centrate under different cultivation modes

Mode and substrate	Lipid (%)	Lipid productivity (mgL ⁻¹ d ⁻¹)	Protein (%)	Protein productivity (mgL ⁻¹ d ⁻¹)	Carbohydrate (%)	Carbohydrate productivity (mgL ⁻¹ d ⁻¹)
Auto BG11	12.7±0.85	10.44±3.09	20.89±0.63	17.4±0.001	28.39±2.46	23.25±6.42
Mixo BG11	15.2±0.99	30.62±2.15	23.15±0.53	46.6±0.83	36.96±4.72	74.46±9.88
Mixo INF	19.5±0.14	14.14±0.20	14.12±0.01	10.24±0.08	24.08±0.21	17.46±0.03
Mixo AC	22.3±0.42	17.2±0.35	15.32±0.23	11.82±0.64	12.95±0.07	10.00±0.45
Mixo AC 500	20.4±0.001	16.68±0.32	13.58±0.54	11.1±0.23	11.16±0.001	9.12±0.18
Mixo AC 1500	15.3±1.27	24.91±3.23	13.77±0.49	22.36±0.25	12.37±0.21	20.10±1.27
Hetero BG11	23.7±0.71	41.19±1.21	23.66±0.03	23.5±0.02	44.50±0.18	77.34±0.27
Hetero INF	22.3±0.42	13.81±1.25	13.79±0.12	8.53±0.68	16.92±1.71	10.43±0.31
Hetero AC	15.1±0.14	11.52±0.64	13.38±0.14	10.20±0.36	16.92±1.03	12.88±0.18

Research on the production of biofuels from microalgal biomass has recently been on the rise due to their ability to accumulate a high lipid content as compared to conventional crops. Figure 3.3 illustrates the microalgal lipid productivities of the experiments in this study. It was seen that amongst synthetic media as a substrate under different cultivation modes, *C. sorokiniana* was able to achieve highest lipid productivity of 41.19 mgL⁻¹d⁻¹ in Hetero BG11 followed by 30.62 mgL⁻¹d⁻¹ in Mixo BG11. Previous studies suggest that cultivations in

heterotrophic systems provide advantages such as consistent and reproducible processes with higher microalgal biomass and lipid production rates, which was apparent in this investigation (Bumbak *et al.*, 2011, Espinosa-Gonzalez *et al.*, 2014, Perez-Garcia *et al.*, 2011).

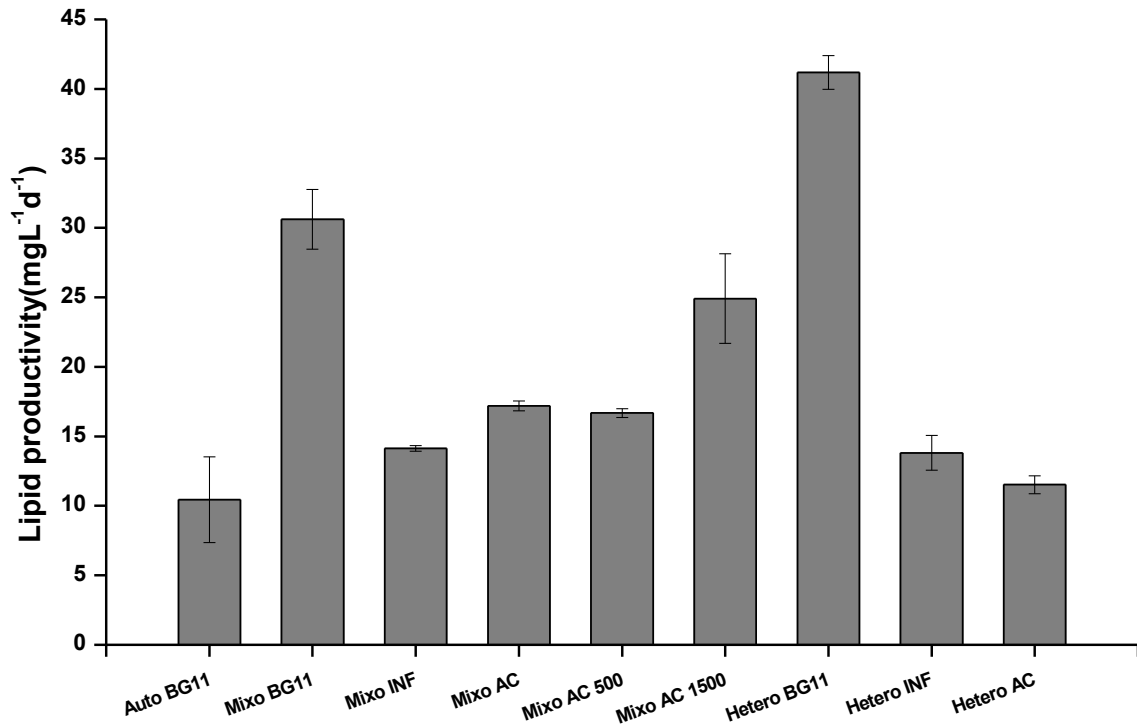


Figure 3.3: Lipid productivities of *Chlorella sorokiniana* cultivated in BG11 medium, influent, anaerobic centrate and urea supplemented anaerobic centrate under different cultivation modes. Data represented as mean \pm SD (n=2).

Amongst the microalgae cultivation in waste substrates under different modes, Mixo AC 1500 appeared to attain the highest lipid productivity of $24.91 \text{ mgL}^{-1}\text{d}^{-1}$. This is an exceptional result as *C. sorokiniana* was able to produce up to 81.35% lipid productivity of that attained in Mixo BG11 as well as maintained high biomass productivity of $162.50 \text{ mgL}^{-1}\text{d}^{-1}$. Mixotrophic anaerobic tank centrate supplemented with 1500 mgL^{-1} urea was found to be a potential mode and substrate replacement to the costly conventional mode and media currently being used and as a potential candidate for the production of microalgal lipids for biodiesel application. However, extensive lipid profiling needs to be conducted to determine its lipid quality for the production of biodiesel.

Although microalgae are a potential feedstock for biofuels, an integrated biorefinery approach is required for the feasibility of biofuels production. Microalgae proteins are expected to play an important role in this approach due to their abundance and amino acid profiles (Spolaore *et al.*, 2006). These proteins have been considered in the application of aquaculture feed, for human consumption, animal feed and cosmetics. In aquaculture, microalgae are important as a natural source of food to species such as fish, shrimps and molluscs (Spolaore *et al.*, 2006). The current study presents protein content and protein productivities attained from the cultivations of *C. sorokiniana* in wastewaters under different cultivation modes and its potential applications.

As seen in Figure 3.4, maximum protein productivities were attained in Mixo BG11 ($46.60 \text{ mgL}^{-1}\text{d}^{-1}$) followed by $23.50 \text{ mgL}^{-1}\text{d}^{-1}$ in Hetero BG11 and $22.36 \text{ mgL}^{-1}\text{d}^{-1}$ in Mixo AC 1500. Approximately 50% of the protein productivity achieved in Mixo BG11 was observed from *C. sorokiniana* in Mixo AC 1500, thus mixotrophic cultivations in wastewater AC with urea

supplementation is a promising mode and substrate for protein production in microalgae. Literature suggests that conventionally grown microalgae have protein quality equal to or even superior to high-quality plant proteins (Spolaore *et al.*, 2006). However, amino acid profiling of proteins from wastewater-grown microalgae is required to evaluate the applications of proteins from *C. sorokiniana* in Mixo AC 1500. Moreover, ethical issues may arise if wastewater-cultivated microalgae are put forth for human consumption due to strict food and safety regulations and thus require extensive risk assessment studies. .

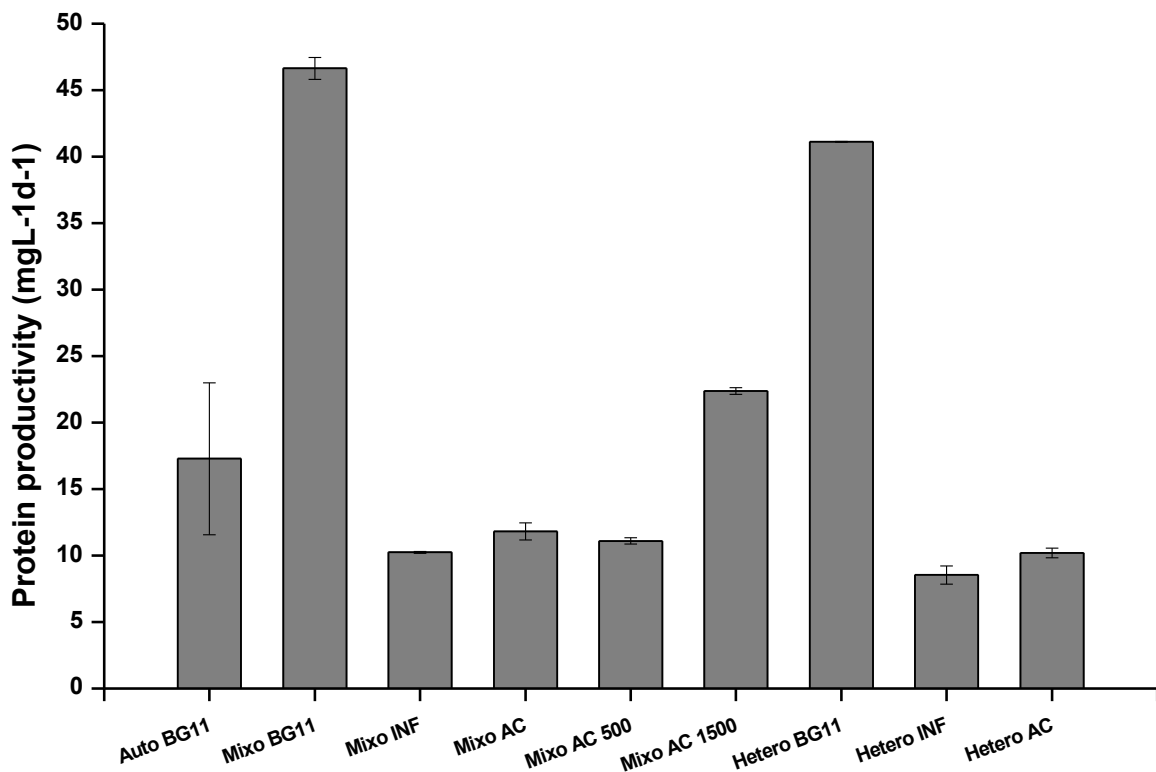


Figure 3.4: Protein productivities of *Chlorella sorokiniana* cultivated in BG11 medium, influent, anaerobic centrate and urea supplemented anaerobic centrate under different cultivation modes. Data represented as mean \pm SD (n=2).

Another useful biochemical component derived from microalgae are carbohydrates. As previously mentioned, microalgae have been an attractive feedstock for biofuels. Microalgal carbohydrates are a promising feedstock for the production of bioethanol (Vieira Salla *et al.*, 2016). Figure 3.5 depicts the carbohydrate productivity of *C. sorokiniana* in wastewaters under different cultivation modes. Figure 3.5 shows the highest carbohydrate productivity of 77.34 mgL⁻¹d⁻¹ in Hetero BG11, amongst synthetic media. Amongst the waste substrates, Mixo INF showed highest carbohydrate content of 24.08% and productivity of 17.46 mgL⁻¹d⁻¹. Mixotrophic cultivation using anaerobic tank centrate showed biomass productivity of 10 mgL⁻¹d⁻¹. The carbohydrate productivity of Hetero AC and Hetero INF were 12.88 and 10.43 mgL⁻¹d⁻¹ respectively. INF as a substrate showed the best potential for carbohydrates production amongst the waste substrate. After supplementation of urea in Mixo AC (Mixo AC 1500) the carbohydrate productivity of 20.1 mgL⁻¹d⁻¹ was achieved.

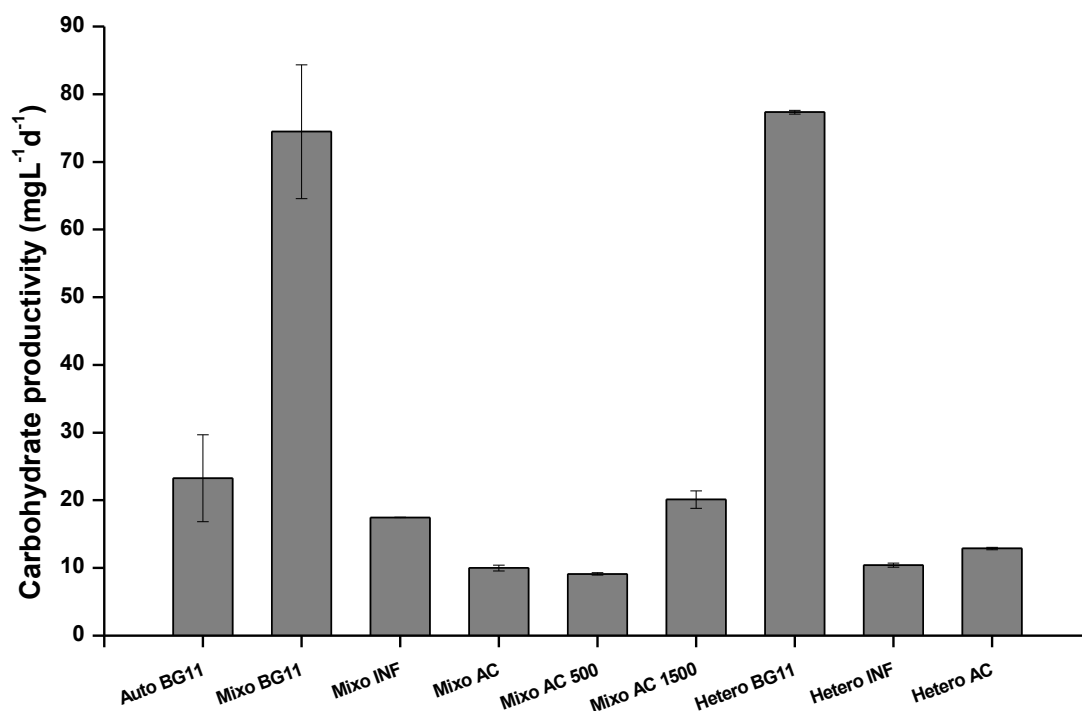


Figure 3.5: Carbohydrate productivities of *Chlorella sorokiniana* in BG11 medium, influent, anaerobic centrate and urea supplemented anaerobic centrate under different cultivation modes. Data represented as mean \pm SD (n=2).

Biochemical analysis of *C. sorokiniana* revealed that mixotrophic mode of cultivation was best suitable for the production of lipids, proteins and carbohydrates. Amongst the waste substrates the AC showed promising potential for microalgal applications based on lipids and proteins, while INF was found to be suitable for carbohydrate based applications of microalgae. The supplementation strategy used for AC substrate increased overall productivities of lipids, proteins and carbohydrates.

Table 3.6 is a compilation of biomass yields, metabolites and nutrient uptake by several microalgal strains cultivated in different types of wastewaters from previous studies in comparison to the present study. It was apparent that the biomass and lipid productivities found in this study were relative to those found in literature using wastewater effluents from different stages of the municipal wastewater treatment process. Present study however implemented a more feasible pretreatment strategy of filtration than previous studies which employed energy intensive processes such as autoclaving and centrifugation. Thus the current study presents a more practical approach that may be applied at scaled up cultivation of microalgae. Moreover, reliable data from literature for the microalgal cultivation in wastewater under different cultivation modes for biomass and metabolite production as well as for nutrient removal is sparse. The comprehensive results of the present study thus suggest that anaerobic centrate (AC) can be effectively used for sustainable microalgal biomass production for various applications simultaneously polishing the AC via nutrients removal. Urea supplementation further adds to the potential of this easily applicable and scalable microalgal cultivation strategy.

Table 3.6: Comparison of biomass productivity, metabolites production and nutrients removal by different microalgae grown in domestic wastewaters from previous studies

Microalgae	Type of wastewater	Pre-treatment	BP* mgL ⁻¹ d ⁻¹	P= productivity in mgL ⁻¹ d ⁻¹ C= content in %			Nutrient removal %			Ref
				L*	PR*	CR*	NH ₄ -N	NO ₃ -N	PO ₄ -P	
Mixed Culture	Domestic Wastewater	Filtration and sterilization	122	C= 18.00-28.5	-	-	89.00	-	70.00	(Mahapatra <i>et al.</i> , 2014)
<i>Chlorella pyrenoidosa</i>	Extract of digested sludge	-	51.8	-	-	-	-	-	-	(Cheung & Wong, 1981)
<i>Scenedesmus</i> sp. ZTY2	Primary effluent supplemented with glucose	Centrifugation and Autoclaving	~ 7.1	C= 69.1	-	-	-	-	35.4	(Zhang <i>et al.</i> , 2013)
<i>Scenedesmus</i> sp. ZTY3	Primary effluent supplemented with glucose	Centrifugation and Autoclaving	~ 7.4	C=55.3	-	-	-	-	32.7	(Zhang <i>et al.</i> , 2013)
<i>Chlorella</i> sp. ZTY4	Primary effluent supplemented with glucose	Centrifugation and Autoclaving	~ 8.8	C=79.2	-	-	-	-	49.90	(Zhang <i>et al.</i> , 2013)
<i>Scenedesmus acutus</i>	Municipal wastewater after primary settling	Autoclaving	107.2	C= 28.3	-	-	92.4	42.9	64.3	(Sacristan de Alva <i>et al.</i> , 2013)
<i>Scenedesmus acutus</i>	Municipal wastewater post activated sludge treatment	Autoclaving	48.1	C= 12.7	-	-	93.6	71.1	66.2	(Sacristan de Alva <i>et al.</i> , 2013)
<i>Scenedesmus obliquus</i>	Municipal secondary effluent	Filtration and Autoclaving	26	C= 31.4 P= 8.00	C= 11.8	-	100.00	-	98.00	(Martínez <i>et al.</i> , 2000)
<i>Botryococcus braunii</i>	Municipal secondary effluent	cellulose acetate membrane filtration	345.6	C=17.85	C= 12.54	-	-	-	-	(Órpez <i>et al.</i> , 2009)
<i>Chlorella sorokiniana</i>	Mixo AC	Filtration (0.45 µm)	77.1	C= 22.3 P= 17.2	C= 15.32 P= 11.80	C= 12.95 P= 10.00	94.29	-	83.3	Present study
<i>Chlorella sorokiniana</i>	Mixo AC 1500	Filtration (0.45 µm)	162.5	C= 15.3 P= 24.91	C= 13.77 P= 22.36	C= 12.37 P= 20.1	-	-	-	Present study

*BP- biomass productivity; L- lipid; PR- protein; CR- carbohydrate

3.4. Conclusion

The physico-chemical characteristics of the screened wastewaters showed promising nitrogen, phosphorous and organic carbon loads in INF and AC wastewaters for microalgal cultivation. Filtration was found to be the most feasible approach as a pretreatment method to reduce the microbial load. Among different wastewaters and cultivation modes, *C. sorokiniana* showed high biomass, lipid, protein and carbohydrate productivities as well as efficient ammonium and phosphate removal rates in Mixo AC. Urea supplementation further enhanced biomass, lipid, protein and carbohydrate productivities. Urea supplemented mixotrophic cultivation of microalgae in AC is an easily applicable and scalable approach for economical microalgal biomass production.

CHAPTER FOUR

EVALUATION OF WASTE ACTIVATED SLUDGE AS A POTENTIAL NUTRIENT SOURCE FOR CULTIVATION OF *CHLORELLA SOROKINIANA*

RAMSUNDAR, P., GULDHE, A., SINGH, P., PILLAY, K., BUX, F. 2017. Evaluation of waste activated sludge as a potential nutrient source for cultivation of *Chlorella sorokiniana*. *Algal Research*, 28, 108–117.

4.1. Introduction

Waste activated sludge (WAS) from the municipal wastewater treatment process is an inevitable by-product. The sludge is generated in large amounts during biological wastewater treatment and is comprised mainly of organic matter, inorganic nutrients and microbial cells. The organic part comprises of approximately 50-55% C, 10-15% N and 1-3% P (Orhon, 1997). Following biological treatment, the sludge is dried and disposed of on landfill sites or sacrificial lands (Tay and Show, 1992). Approximately 40–60% of wastewater treatment plant's expenses are directly related to WAS treatment and disposal (Kavitha *et al.*, 2014).

Due to its favourable nutrient composition and when stabilized through a composting process, WAS has been successfully utilized as organic fertilizers or soil additives. The WAS comes to the forefront of microalgal biotechnology as it could be used as a potential nutrient source for microalgal cultivation. Utilizing WAS as a nutrient source for microalgae cultures could minimize the dependence on synthetic fertilizers reducing overall cost of production.

Utilizing final effluent as a liquid medium will minimize the use of fresh water. Overall, this could be a sustainable waste management strategy which provides environmental benefit in terms of re-cycling of nutrients, primarily N and P, from sludge and final effluent by incorporation into microalgal biomass. Moreover, this technique may reduce the effects of sludge disposal on the environment, whilst achieving microalgal biomass with potential applications in fuel and feed industries. Currently, to our knowledge, there is no research published on the utilization of municipal dried waste activated sludge as a nutrient medium for microalgal biomass production and its applied value.

Microalgal strain *C. sorokiniana* was selected in the present work for its adaptability towards wastewater and various cultivation modes (Kim *et al.*, 2013). The objectives of this aspect of the study was to (1) physico-chemically and biologically characterize municipal dried WAS and FE, (2) to develop pre-treatment methods for effective nutrient release from WAS supplemented into FE, (3) different cultivation modes such as mixotrophic and heterotrophic were evaluated for microalgal biomass production (4) urea supplementation was studied to enhance biomass productivity and (5) microalgal lipids, proteins and carbohydrates yields were determined.

4.2. Materials and methods

4.2.1. Microalgal culture

Chlorella sorokiniana was isolated from the Durban region, KwaZulu-Natal, South Africa, purified by subsequent sub-culture using streak plate method and was maintained in sterilized Blue-Green (BG11) media (Ramanna *et al.*, 2014). Antibiotic (ampicillin $1 \mu\text{g mL}^{-1}$) was added to the media to reduce susceptibility to bacterial contamination. Exposure to an irradiance of approximately $120 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, light:dark-cycle of 16:8 h, at room temperature ($25 \pm 1^\circ\text{C}$) and orbital shaking (Labcon, South Africa) at 120 rpm was provided to the *C. sorokiniana* stock.

4.2.2. Collection and characterization of municipal waste substrates

Sun-dried municipal WAS and FE (in 25 L batches) were collected from the Kingsburgh Wastewater Treatment Works in Durban, South Africa ($S30^\circ04.29'$; $E30^\circ51.26'$). Prior to use, the WAS was ground in a laboratory scale blender (WARING Commercial Blender, HGB250, USA) and sieved ($710 \mu\text{m}$) to obtain a powder form. The FE was characterized for physico-chemical as well as biological parameters. The pH and temperature were measured using pH meter (Thermo Scientific™ Orion™ DUAL STAR™ Meter, UK). Turbidity was measured using a turbidimeter (2100P-HACH). Nitrate, $\text{NO}_2\text{-N}$, $\text{NH}_4\text{-N}$, and $\text{PO}_4\text{-P}$ concentrations were determined using a colorimetric method with the (Thermo Scientific™ Gallery™ Automated Photometric Analyzer, Germany). Chemical oxygen demand was determined using colorimetric standard methods (Eaton *et al.*, 2005). Metals quantification were determined

using a modified method by Suthar *et al.* (2009) and Agilent 4200 Microwave Plasma-Atomic Emission Spectrometer (MP-AES) was used to analyze samples. The FE was subjected to biological analysis via heterotrophic plate counts (HPC) on R-2A agar plates using the spread plate method with incubation at 30°C for 7 days (Eaton *et al.*, 2005).

4.2.3. Pre-treatment and nutrient release of waste activated sludge in final effluent

Prior to pre-treatment, WAS powder was added to FE at a concentration of 10 gL⁻¹. The WAS + FE was subjected to orbital shaking at 200 rpm for 1 hr. The FE+WAS was filtered with Whatman no.1 filter paper and the filtrate (RAW) was used as a control for microalgal cultivation studies. Chemical, physical and thermal pre-treatments were applied to WAS + FE. There were two approaches to the chemical pre-treatment: (1) to increase the pH of the WAS + FE to pH 11, 5 M NaOH was used and (2) to lower the pH to pH 2, 5 M HCl was used (Xu *et al.*, 2015). Changes in pH were monitored with a pH meter (Thermo Scientific™ Orion™ DUAL STAR™ Meter, UK). The chemically treated WAS + FE were subjected to orbital shaking at 200 rpm for 1 h. Physical pre-treatment involved sonication of the WAS + FE. Sonication was conducted at 20 W for 10 min. For thermal pre-treatment, WAS + FE was subjected to autoclaving at 121°C for 15 min in accordance with Zhou *et al.* (2012b). Microwave digestion (Milestone S.R.L., Italy, output power 1200 W) was conducted at a temperature of 100°C for 10 min at 600 W power (Byun *et al.*, 2014).

The WAS + FE was physico-chemically and biologically characterized before and after exposure to pre-treatment methods to determine the most effective and feasible pre-treatment method in releasing nutrients as well as reducing the microbial load of the WAS + FE for

microalgal cultivation. All pre-treated WAS + FE were subjected to filtration with Whatman no.1 filter paper before characterizations.

4.2.4. Microalgal cultivation

4.2.4.1. Microalgae cultivation in synthetic media

BG11 medium was used to cultivate *C. sorokiniana*. For prevention of bacterial contamination, ampicillin ($1 \mu\text{g mL}^{-1}$) was added to all synthetic media. The cultivation experiments were conducted in 1 L Erlenmeyer flasks with a working volume of 500 mL BG11 media with the pH adjusted to 7.0 at the time of inoculation. The pH adjustments were done using either 1 M H_2SO_4 or 1 M NaOH. The cultures were subjected to incubation at room temperature with orbital shaking at 120 rpm. For photoautotrophic cultivation, illumination of approximately $120 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ was provided with a light:dark-cycle of 16:8 h (Singh *et al.*, 2016a). For mixotrophic and heterotrophic cultivations, 5 gL^{-1} glucose was used as a carbon source with and without illumination, respectively (Kim *et al.*, 2013).

4.2.4.2. Microalgal cultures in waste activated sludge dissolved in final effluent

Post pre-treatment of waste substrates, *C. sorokiniana* was inoculated into 500 mL RAW WAS + FE and pH 2 treated WAS + FE (pH 2 WAS + FE) in 1 L Erlenmeyer flasks. The cultures were incubated at room temperature and subjected to orbital shaking at 120 rpm. Illumination of approximately $120 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ with a light:dark-cycle of 16:8 h were

provided for mixotrophic cultures whilst heterotrophic cultures were subjected to dark conditions by covering the Erlenmeyer flasks with aluminium foil (Ramsundar *et al.*, 2017). Experimentation for mixotrophic and heterotrophic cultivations were carried out in duplicate and over a period of 14 and 8 days respectively. Nitrate, NH₄-N, PO₄-P and COD concentrations were determined prior to and post cultivation with methods described under section 4.2.2.

4.2.4.3. Nitrogen supplementation for microalgal cultivation

For the subsequent growth studies of *C. sorokiniana* and to increase biomass productivities, urea (250 mgL⁻¹, 500 mgL⁻¹ and 1500 mgL⁻¹) was supplied as a nitrogen source to pH 2 WAS+FE. Experimentation was conducted over a duration of 14 days for the mixotrophic cultures as these were the optimal conditions observed for high biomass productivities. BG11 medium (amended with 5 gL⁻¹ glucose) and filtered pH 2 WAS + FE without urea supplementation were used as positive and negative controls respectively.

4.2.5. Analytical methods

To monitor microalgal growth, daily OD₆₈₀ readings, biomass expressed in gL⁻¹ using DCW measurements, photosynthetic performance of microalgae using PAM Fluorometry and metabolites production (lipid, protein and carbohydrates) were determined according to the analytical methods followed in Chapter 3, section 3.2.5.

4.2.6. Statistical analysis

Minitab statistical software was used for statistical analysis of data. Significance of results and differences amongst the different pre-treatments were evaluated for duplicate set of data by using one-way analysis of variance (ANOVA). Posthoc Tukey's test ($P = 0.05$) was used for comparisons amongst the various means. In tables, the data were represented as Mean \pm SD (standard deviation), while in figures; SD values were represented as error bars.

4.3. Results and discussion

4.3.1. Screening and pre-treatment effects on waste substrate nutrient recovery and bacterial load

Orbital shaking of RAW at 200 rpm for 1 hr showed considerable potential in releasing WAS COD and nutrients into FE. The COD concentration increased from $55.53 \pm 0.29 \text{ mgL}^{-1}$ in FE to $338.91 \pm 0.45 \text{ mgL}^{-1}$ after addition of WAS (Table 4.1). Thus WAS can be used as a carbon source for mixotrophic and heterotrophic microalgal cultivations. Moreover, $\text{NH}_4\text{-N}$ increased to approximately 2 fold the concentration of that in FE. Nitrate concentration increased from $0.03 \pm 0.01 \text{ mgL}^{-1}$ to $6 \pm 0.02 \text{ mgL}^{-1}$ and $\text{NO}_2\text{-N}$ considerably increased from $0.07 \pm 0.01 \text{ mgL}^{-1}$ to $30 \pm 0.11 \text{ mgL}^{-1}$ and up to $7.57 \pm 0.06 \text{ mgL}^{-1}$ of $\text{PO}_4\text{-P}$ solubilized into FE. WAS dissolved in FE showed potential to provide microalgae-essential nutrients for biomass production. However, pre-treatments were necessary in order to enhance the nutrient release as well as for the reduction of bacterial load ($9.35 \times 10^6 \text{ CFU mL}^{-1}$) in RAW. Wastewater contains microorganisms that may cause constraints during microalgal cultivations. Various pre-

treatment methods used in this study showed varying influence on the nutrients in WAS dissolved in FE. Table 4.1 depicts the characteristics of WAS before and after being subjected to thermal, physical and chemical pre-treatments.

Table 4.1: Screening and effect of thermal, physical and chemical pre-treatments on physico-chemical characteristics and bacterial load of waste substrates

Parameter	FE	RAW	Autoclaved WAS + FE	Microwaved WAS + FE	Sonicated WAS + FE	pH 2 WAS +FE	pH 11 WAS + FE
pH	7.42±0.01	6.93±0.03	6.78±0.05	4.49±0.08	7.9±0.01	2.05±0.02	11.02±0.01
Temperature (°C)	24.95±0.07	25.1±0.01	25.1±0.01	25.25±0.07	25.2±0.07	25.1±0.01	24.95±0.07
COD (mgL ⁻¹)	55.53±0.29	338.91±0.45	1103.79±0.35	1067.93±0.59	221.67±1.05	207.78±0.28	388.42±0.36
NH ₄ -N (mgL ⁻¹)	7.51±0.03	13.7±0.03	1.71±0.04	1.38±0.03	11.39±0.11	13.36±0.24	15.52±0.22
NO ₃ -N (mgL ⁻¹)	0.03±0.01	6±0.02	2.14±0.04	6.23±0.01	5.78±0.21	5±0.01	4.03±0.07
NO ₂ -N (mgL ⁻¹)	0.07±0.01	30±0.11	0.04±0.01	28±0.03	27±0.01	40±0.01	36±0.08
PO ₄ -P (mgL ⁻¹)	0.18±0.01	7.57±0.06	8.68±0.06	12.27±0.25	4.83±0.06	22.86±0.41	17.08±0.28
Cu ²⁺ (mgL ⁻¹)	0.07±0.03	0.52±0.06	0.14±0.01	0.16±0.04	0.16±0.03	0.33±0.04	0.22±0.03
Co ²⁺ (mgL ⁻¹)	0.65±0.05	0.03±0.03	0.82±0.07	1.04±0.02	1.18±0.08	0.91±0.16	0.99±0.04
Fe ³⁺ (mgL ⁻¹)	1.07±0.02	1.38±0.38	1.41±0.02	2.30±0.05	1.99±0.01	2.89±0.08	1.31±0.03
Mo ⁶⁺ (mgL ⁻¹)	0.02±0.01	0.04±0.01	0.05±0.01	0.03±0.01	0.02±0.01	0.02±0.01	0.02±0.01
Mn ²⁺ (mgL ⁻¹)	0.08±0.01	0.18±0.05	0.06±0.01	0.15±0.01	0.09±0.01	0.77±0.01	0.04±0.01
K ⁺ (mgL ⁻¹)	7.88±0.09	1.21±0.20	22.46±0.30	25.69±0.25	27.97±0.38	23.74±0.21	19.21±0.36
Mg ²⁺ (mgL ⁻¹)	-	0.26±0.38	7.20±0.39	13.51±1.53	7.24±0.62	18.33±3.02	2.08±0.12
Zn ²⁺ (mgL ⁻¹)	0.25±0.03	6.50±0.55	1.69±0.04	0.18±0.01	1.04±0.03	1.83±0.01	0.13±0.02
Na ⁺ (mgL ⁻¹)	14.98±12.24	31.95±28.41	19.04±20.44	24.48±6.57	7.95±22.76	25.94±4.74	20.42±6.53
TSS (mgL ⁻¹)	45±0.01	35±0.01	25±0.01	75±7.07	25±0.01	66±1.41	190±14.14
VSS (mgL ⁻¹)	0.08±0.01	0.09±0.00	0.07±0.01	0.09±0.01	0.03±0.01	0.09±0.01	0.2±0.01
Turbidity (NTU)	3.72±0.36	40.80±1.41	11.9±0.42	34.05±0.35	28.5±0.28	1.66±0.01	57.35±1.48
HPC (CFU mL ⁻¹)	2.62 × 10 ⁶	9.35 × 10 ⁶	-	-	5.95 × 10 ³	2.95 × 10 ³	1.58 × 10 ⁷
Power consumption (kWh)	-	-	0.63	0.1	0.03	0.03	0.03

4.3.1.1. Effect of thermal pre-treatment on waste substrate

As seen in Table 4.1, thermal pre-treatments by autoclaving and microwave digestion were most effective in the reduction of bacterial load from WAS + FE. Amongst all pre-treatments applied, the thermal pre-treatments were most effective in COD recovery from sludge (1103.79±0.35 mgL⁻¹ by autoclaving and 1067.93±0.59 mgL⁻¹ by microwave). Although thermal pre-treatments were effective in sterilizing the WAS + FE as well as in high COD release, COD at such high concentrations may negatively influence microalgal growth. The tolerance of high COD has not yet been explored for *C. sorokiniana* cultures. A study by Gupta *et al.* (2016a) states that wastewaters with high COD concentrations may inhibit microalgal growth. Thus it is important to delve into optimizing COD concentrations that *C. sorokiniana* is able to withstand if thermal pre-treatments are employed.

Results of the present study further revealed that autoclaving hampered with the nutrient recovery process of WAS into FE with significant reductions in microalgae-essential nutrients that would have been released without any pre-treatment (RAW). Although PO₄-P recovery increased by 12.79% with autoclaving, reductions of 87.59% NH₄-N, 64.33% NO₃-N and 99.87% NO₂-N were observed. An investigation by Sriram and Seenivasan (2012) confirmed that autoclaving hampers with the nutrients present in wastewaters and thus may affect the microalgal growth. The decrease in NH₄-N can be attributed to volatilization during autoclaving (Ho *et al.*, 2013). Exogenous chemicals are needed to be supplied in such cases for the growth of microalgae however, it adds excessive costs. Moreover, autoclaving showed power consumption of 0.63 kWh which was highest among the selected pre-treatment methods. Microwaving resulted in power consumption of 0.1 kWh. Both these thermal pre-treatments

are energy intensive for their feasible application at commercial scale, despite their efficiencies in COD recovery and elimination of undesired microbes.

4.3.1.2. Effect of physical pre-treatment on waste substrate

Sonication was the physical pre-treatment investigated for WAS + FE nutrient recovery and bacterial reduction. After 10 min of sonication the COD increased from $55.53 \pm 0.29 \text{ mgL}^{-1}$ in FE to $221.67 \pm 1.05 \text{ mgL}^{-1}$ in WAS + FE. The increase in COD was due to the disintegration of WAS and microbial cells resulting in release of intracellular substances. A previous study using ultrasonic pre-treatment described that during treatment of WAS, cavitation is triggered which leads to sudden and violent collapsing of numerous microbubbles (Pilli *et al.*, 2011). These bubble implosions generate powerful hydro-mechanical shear forces which destroy extracellular polymeric substances and cell walls of WAS in the liquid surrounding the bubbles (Khanal *et al.*, 2007). Thus sonication pre-treatment disintegrated flocs of WAS and microbial cells which consequently discharge nutrients from the WAS solids into the aqueous phase (FE). This also explains the reduction of microbial load from $9.35 \times 10^6 \text{ CFU mL}^{-1}$ in RAW to $5.95 \times 10^3 \text{ CFU mL}^{-1}$ in sonicated WAS + FE.

Although sonication pre-treatment was able to recover amounts of COD and microalgae-essential nutrients ($11.39 \pm 0.11 \text{ mgL}^{-1} \text{ NH}_4\text{-N}$ and $4.83 \pm 0.06 \text{ mgL}^{-1} \text{ PO}_4\text{-P}$), these concentrations were lower in comparison to COD and nutrients recovered in RAW which was without pre-treatment ($13.7 \pm 0.03 \text{ mgL}^{-1} \text{ NH}_4\text{-N}$ and $7.57 \pm 0.06 \text{ mgL}^{-1} \text{ PO}_4\text{-P}$). This could be due to the short treatment time. Increasing the time of treatment from 10 min may assist in efficient nutrient recovery. Moreover, extreme conditions (heating) of samples caused by the

sudden and violent collapsing of the microbubbles may have suppressed efficient nutrient recovery or the heat may have volatilized essential nutrients during treatment. A cooling mechanism will be required to enhance effectiveness of sonication as a pre-treatment strategy, however it will add to the cost of sonication pre-treatment.

4.3.1.3. Effect of chemical pre-treatment on waste substrate

For chemical pre-treatments, pH is the main influencing factor. In this study, alkaline and acidic conditions were applied to the WAS + FE. Table 4.1 shows the trends of the COD and nutrient release as well as the bacterial reduction in WAS + FE under different pH values (pH 2 and pH 11). The results show excellent WAS disintegration and solubilisation of COD and nutrients from both alkaline and acidic pre-treatments. The COD release at pH 11 was considerably higher ($388.42 \pm 0.36 \text{ mgL}^{-1}$) than at pH 2 ($207.78 \pm 0.28 \text{ mgL}^{-1}$). Thus a clear increase of COD was seen with increased pH. Kim *et al.* (2009) and Li *et al.* (2012) observed similar trends in COD recovery from sludge when alkaline conditions (addition of NaOH) were provided.

In terms of nutrient release, $\text{NH}_4\text{-N}$ concentrations in both pH 2 ($13.36 \pm 0.24 \text{ mgL}^{-1}$) and pH 11 ($15.52 \pm 0.22 \text{ mgL}^{-1}$) treated WAS + FE were almost similar to the $\text{NH}_4\text{-N}$ recovered in RAW ($13.7 \pm 0.03 \text{ mgL}^{-1}$). However in comparison to RAW, considerable increases of $\text{NO}_2\text{-N}$ and $\text{PO}_4\text{-P}$ recovery were obtained from acid pre-treatment which were 25% and 66.89% respectively. A study by Stark *et al.* (2006) observed high phosphate release of up to 85% from dried sludge when using acid leaching, while they observed 70% phosphate release using base leaching. Another investigation by Xu *et al.* (2015) showed that phosphorous recoveries were

36.2% and 12.4% at pH 2 and pH 11 respectively. Thus acid (pH 2) pre-treatment was found to be efficient in nutrient release, especially in PO₄-P release which is vital for microalgal growth. The extreme alkaline conditions (pH 11) possibly inhibited PO₄-P release as at high pH PO₄ precipitates out of solution as struvite (NH₄MgPO₄.6H₂O) (Münch and Barr, 2001).

The acid pre-treatment was found to be the most effective chemical pre-treatment in reducing the bacterial load in RAW from 9.35×10^6 CFU mL⁻¹ to 2.95×10^3 CFU mL⁻¹ in pH 2 WAS + FE. Alkaline pre-treatment (pH 11 WAS + FE) however allowed for bacteria to thrive and was found to contain the maximum bacterial load of 1.58×10^7 CFU mL⁻¹ which could be detrimental to microalgal cultures. The power consumption of both the pre-treatment methods was 0.03 kWh which was much lower than the thermal pre-treatments and comparable to sonication (Table 4.1). Taking these crucial factors into consideration, pH 2 was selected as the most effective pre-treatment method for nutrient release and bacterial reduction. However, due to extreme acid conditions, pH 2 WAS + FE was neutralized (pH 7) with 5 M NaOH before inoculation of *C. sorokiniana*.

4.3.2. Effect of pre-treated waste substrate on biomass production and photosynthetic performance of microalgae under different cultivation modes

Table 4.2 and Figure 4.1 are representations of the primary objective of this study which was to assess the suitability of WAS in FE as a growth medium for Mixo and Hetero *C. sorokiniana* cultivation. The rETR is a determination of the rate of linear electron transport through PS II and F_v/F_m is the quantum efficiency of PS II which indicates the photosynthetic performance of microalgae. These parameters showed the adaptability and physiological health

of *C. sorokiniana* grown under different modes of cultivation. Biomass productivities obtained from mixotrophic RAW WAS + FE was $79.64 \pm 3.54 \text{ mgL}^{-1}\text{d}^{-1}$ which was comparable to biomass obtained from autotrophic synthetic medium ($85 \pm 0.01 \text{ mgL}^{-1}\text{d}^{-1}$ in Auto BG11). An improved Fv/Fm value (0.62 ± 0.01) and rETR value (39.75 ± 0.07) were also observed when the culture was grown under Mixo RAW conditions (Table 4.2). The biomass productivity was further improved when acid pre-treatment was employed. The biomass obtained in Mixo pH 2 WAS + FE was $128.57 \pm 3.03 \text{ mgL}^{-1}\text{d}^{-1}$. Even though the COD concentration in RAW ($338.91 \pm 0.45 \text{ mgL}^{-1}$) was higher than in pH 2 WAS + FE ($207.78 \pm 0.28 \text{ mgL}^{-1}$), the pre-treated waste substrate showed greater biomass productivity. Miao *et al.* (2016) also successfully cultivated *Chlorella* sp. in domestic wastewater under mixotrophic conditions.

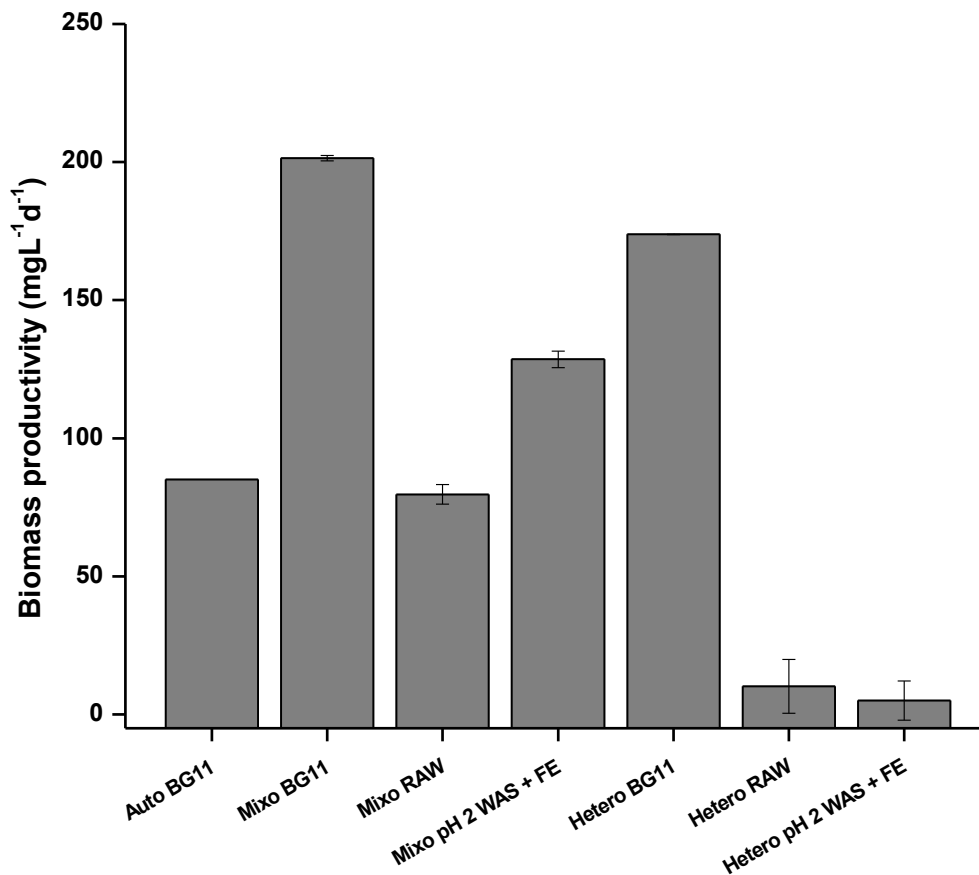


Figure 4.1: Biomass productivities of *Chlorella sorokiniana* cultivated in untreated and pre-treated dried waste activated sludge in final effluent and in BG11 under different modes of cultivation. Data represented as mean \pm SD (n=2).

For heterotrophic cultivations, both RAW and pre-treated WAS + FE showed biomass productivities in the range of 5-10 mgL⁻¹d⁻¹, which were much lower than the mixotrophic cultivation. Results indicate that the microalgae cultured heterotrophically struggled to compete with undesired microbes within the WAS + FE and suggest that heterotrophic microalgal cultivation mode was not suitable while using the selected nutrient source. Reasoning behind this could be because the heterotrophic bacteria dominated the nutrient uptake in dark conditions. Thus heterotrophic mode was eliminated for further investigation.

Although the mixotrophic cultivations showed promise over the heterotrophic cultures in WAS + FE, the biomass productivities attained were substantially lower in comparison to the biomass productivities obtained in synthetic media under mixotrophic mode. In this study, Mixo BG11 showed a biomass productivity of $201.4 \pm 1.01 \text{ mgL}^{-1}$ which was much higher than the Mixo pH 2 WAS + FE ($128.57 \pm 3.03 \text{ mgL}^{-1}\text{d}^{-1}$). Moreover, the photosynthetic performance of *C. sorokiniana* was also investigated to ensure the suitability of selected medium for different modes of cultivation (Table 4.2). The Fv/Fm value in Mixo pH 2 WAS + FE was 0.52 in late log phase which was comparable to the Fv/Fm value of 0.58 observed in Auto BG11.

Table 4.2: Biomass, biomass productivity and photosynthetic performance (Fv/Fm and rETR) of *Chlorella sorokiniana* cultivated in RAW and pre-treated waste substrates

Mode and Substrate	Biomass (mgL^{-1})	Biomass Productivity ($\text{mgL}^{-1}\text{d}^{-1}$)	Fv/Fm late log	rETR late log
Auto BG11	1165 ± 0.01	85 ± 0.01	0.58 ± 0.01	35.05 ± 0.07
Mixo BG11	2820 ± 0.01	201.4 ± 1.01	0.69 ± 0.05	22.6 ± 0.28
Mixo RAW	1115 ± 49.5	79.64 ± 3.54	0.62 ± 0.01	39.75 ± 1.76
Mixo pH 2 WAS +FE	1800 ± 42.43	128.57 ± 3.03	0.52 ± 0.01	20.6 ± 0.85
Mixo pH 2 WAS + FE 250	2825 ± 240.72	201.79 ± 24.24	0.52 ± 0.01	52.4 ± 0.00
Mixo WAS + FE 500	3162.5 ± 473.91	225.89 ± 47.73	0.63 ± 0.01	55.45 ± 0.92
Mixo WAS + FE 1500	4182.5 ± 554.15	298.75 ± 55.81	0.50 ± 0.15	54.6 ± 0.00
Hetero BG11	1390 ± 0.71	173.81 ± 0.09	-	-
Hetero RAW	78 ± 4.24	10.12 ± 9.75	-	-
Hetero pH 2 WAS + FE	40.05 ± 56.5	5.01 ± 7.06	-	-

3.3. Nutrient and organic carbon removal by microalgae

Nutrient removal efficiencies of *C. Sorokiniana* in Mixo RAW, Mixo pH 2 WAS + FE, Hetero RAW and Hetero pH 2 WAS + FE are represented in Table 4.3. As seen in Table 4.1, most of N content in the RAW is in the form of NH₄-N. In Table 3 efficient NH₄-N removal were observed by *C. sorokiniana* in Mixo RAW and Mixo pH 2 WAS + FE at rates of 97.8% and 97.78% respectively. However heterotrophic conditions demonstrated NH₄-N removal rates of 9.6% from Hetero RAW and 8.89% from Hetero pH 2 WAS + FE. Ammonia removal is caused by direct NH₄-N utilization by microalgae and stripping of NH₃ (Tam and Wong, 1990). Studies have suggested that alkaline conditions at raised temperatures allow for stripping of NH₃ (Reeves, 1972). In the present study, the temperature was constant at 25±1°C, therefore NH₃ stripping may have been insignificant and the reduction in ammonia could largely be attributed to absorption by *C. Sorokiniana*. Moreover, microalgal tolerance to NH₄-N is a strain specific response (Morales-Amaral *et al.*, 2015), thus *C. Sorokiniana* evidently showed pronounced NH₄-N uptake specifically under mixotrophic conditions.

Table 4.3: *Chlorella sorokiniana* nutrient and chemical oxygen demand removal efficiencies in untreated (RAW) and pH 2 treated waste activated sludge in final effluent (WAS + FE) under mixotrophic and heterotrophic modes of cultivation

Mode + Substrate	NH ₄ -N (%)	NO ₃ -N (%)	NO ₂ -N (%)	PO ₄ -P (%)	COD Removal (%)
Mixo RAW	97.8	16.67	10	94.55	28.47
Mixo pH 2 WAS + FE	97.78	60	25	94.91	47.50
Hetero RAW	9.6	16.67	6.67	1.36	86.01
Hetero pH 2 WAS + FE	8.89	20	7.50	7.41	52.41

Removal rates of $\text{NO}_3\text{-N}$ were observed to have been higher in pH 2 WAS + FE in both Hetero and Mixo cultures than in RAW cultures. However, Mixo pH 2 WAS + FE showed considerable increase in the removal rate of $\text{NO}_3\text{-N}$ (60%). According to literature, ammonium and nitrate are known to be the primary sources of N for many organisms (Bloom *et al.*, 1992). Studies found that *Chlorella* sp. could successfully utilize ammonium and nitrate (Wang *et al.*, 2010b). The current investigation demonstrated that $\text{NH}_4\text{-N}$ was preferred over $\text{NO}_3\text{-N}$ and $\text{NO}_2\text{-N}$ in mixotrophic conditions. Despite lower $\text{NO}_3\text{-N}$ and $\text{NO}_2\text{-N}$ concentrations than $\text{NH}_4\text{-N}$ concentrations within the WAS + FE, $\text{NO}_3\text{-N}$ was the preferred N source over $\text{NH}_4\text{-N}$ and $\text{NO}_2\text{-N}$ for cultures grown heterotrophically.

Generally, microalgae assimilate $\text{NH}_4\text{-N}$ (reduced N form), $\text{NO}_3\text{-N}$ and $\text{NO}_2\text{-N}$ (oxidized N forms) by means of different metabolic processes. Literature explains that nitrate reductase and nicotinamide adenine dinucleotide (NADH) are accountable for the reduction of $\text{NO}_3\text{-N}$ to $\text{NO}_2\text{-N}$ by the transfer of two electrons (Ge and Champagne, 2015). This is followed by nitrite reductase and ferredoxin (Fd) reduction of $\text{NO}_2\text{-N}$ to $\text{NH}_4\text{-N}$ by the transfer of a total of six electrons throughout the reaction. Ultimately, prior to amino acid incorporation of intracellular fluid, all forms of inorganic N are reduced to ammonium (Ge and Champagne, 2015). Thus it can be deduced that when *C. Sorokiniana* was cultivated under Mixo conditions, $\text{NH}_4\text{-N}$ rather than $\text{NO}_3\text{-N}$ was preferred for growth due to the minimal energy requirement for its assimilation as redox reactions were not required.

The preference of $\text{NO}_3\text{-N}$ in Hetero cultures could be attributed to the deprivation of illumination. Microalgal cells would have resorted to the preferential utilization of carbon for anaplerotic reactions rather than photosynthetic reactions for microalgal growth (Eustance *et*

al., 2013). This would explain the higher COD removal rates in heterotrophic cultures than mixotrophic cultures as illustrated in Table 4.4. *C. Sorokiniana* was able to remove 86.01% COD in Hetero RAW and 52.41% COD in Hetero pH 2 WAS + FE which were higher than COD removal rates achieved by mixotrophic cultures. Several studies demonstrated similar trends and suggest that higher amounts of COD are capable of being assimilated by microalgae under light deprivation and that various carbon sources other than CO₂ can be utilized (Zhou *et al.*, 2012a). Kim *et al.* (2013) confirms this by the cultivation of *C. sorokiniana* with COD removal rates of 70% and 65% under Hetero and Mixo modes respectively.

Results for PO₄-P removal showed a substantial removal rate under mixotrophic conditions. Phosphate removal was 94.55% in Mixo RAW and 94.91% in Mixo pH 2 WAS + FE. Mahapatra *et al.* (2014) in their study, attained 78% PO₄-P removal by microalgal consortia mixotrophically cultivated in municipal wastewater. Microalgae may accumulate intracellular phosphorous reserves as polyphosphate granules. This reserve can be utilized when phosphate is exhausted within the culture medium. This phenomenon is known as luxury uptake and is beneficial for prolonged cultivations as microalgae may indulge on phosphate from their phosphorous storage. Phosphate removal rates for heterotrophic cultures were considerably low. Only 1.36% and 7.41% PO₄-P was removed by *C. Sorokiniana* in Hetero RAW and Hetero pH 2 WAS + FE respectively. Thus mixotrophic conditions has more potential in removal of PO₄-P than heterotrophic conditions when WAS + FE is utilized as a growth medium for microalgae.

4.3.3. Supplementation with exogenous nitrogen

Reports have shown that nitrogen is the most important nutrient affecting biomass growth and lipid productivities of various microalgae (Singh *et al.*, 2016b). According to the nutrient removal studies in this work, ammoniacal nitrogen was preferred over nitrates for mixotrophic cultures with removal rates of up to 97.8% ammonia and 25% nitrate. Studies by Lizzul *et al.* (2014) and Ramanna *et al.* (2014) suggest that *C. sorokiniana* was grown more rapidly with urea and ammonium than with nitrate. Thus for further investigation to enhance the growth of *C. Sorokiniana* in Mixo pH 2 WAS + FE, urea was selected as a cheap source of exogenous nitrogen.

Figure 4.2 and Table 4.2 report the biomass productivities of *C. sorokiniana* cultivated in Mixo pH 2 WAS + FE with urea supplementation. The supplied concentrations of urea were 250 mgL⁻¹, 500 mgL⁻¹ and 1500 mgL⁻¹ and from the results it was observed that the biomass productivities increased with increasing concentrations of urea. The highest biomass productivity of 298.75±55.81 mgL⁻¹d⁻¹ was achieved from Mixo pH 2 WAS + FE 1500 which was higher than the biomass productivity of Mixo BG11 (201.4±1.01 mgL⁻¹d⁻¹). Nitrogen (urea) supplementation also resulted in an enhanced photosynthetic performance. Both Fv/Fm ratio and rETR values were high for all nitrogen supplemented conditions (Table 2). A high rETR value of 52.4±0.00, 55.45±0.92, 54.6±0.00 was observed when Mixo WAS + FE was supplemented with the urea concentration of 250 mgL⁻¹, 500 mgL⁻¹ and 1500 mgL⁻¹ respectively as compared to rETR values (20.6±0.85) under WAS + FE without any nitrogen supplementation. After a cultivation period of 14 days, the final biomass concentrations of 4182.5±554.15 mgL⁻¹ and 2820±0.01 mgL⁻¹ were achieved in Mixo pH 2 WAS + FE 1500 and

Mixo BG11 respectively. During supplementation the hydrolysis of urea to ammonium results in increased free ammonium availability for easy microalgal uptake.

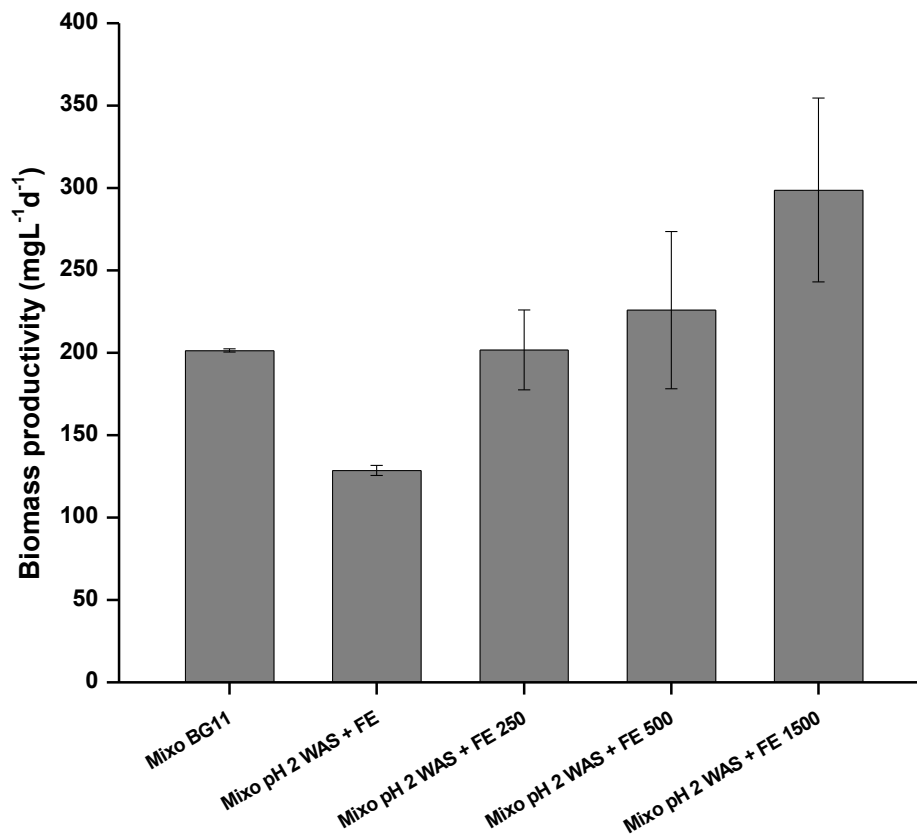


Figure 4.2: Biomass productivities of *Chlorella sorokiniana* cultivated in pre-treated dried waste activated sludge in final effluent with urea supplementation and in BG11 under different modes of cultivation. Data represented as mean \pm SD (n=2).

The results of the current investigation suggest that *C. sorokiniana* was able to thrive despite the presence of the microbial communities within the waste substrate with urea supplementation (1.28×10^8 CFU mL⁻¹ post cultivation in Mixo WAS + FE 1500). Gonzalez and Bashan (2000) cultivated *Chlorella vulgaris* in Auto conditions and found that bacteria had increased the microalgal population. On the other hand, certain strains of bacteria are known to release compounds that are algicidal or inhibitory which may reduce microalgal growth (Munoz and Guieysse, 2006). The strategy developed in this study where a waste substrate is supplemented with a cheap N source has the potential to make the microalgal cultivation process economically viable.

4.3.4. Biochemical constituents

The applications of microalgal biomass in biofuels and bio-products production are highly dependent upon the biochemical constituents (lipids, proteins and carbohydrates). The concentration of these constituents are highly strain specific and are subject to change with change in cultivation conditions and composition of nutrients in the media. Table 4.4 shows the changes in cell constituents when *C. sorokiniana* was cultivated in waste substrates in comparison to synthetic media under different cultivation modes.

Table 4.4: Biochemical composition of *Chlorella sorokiniana* in BG11 medium, untreated, treated and urea supplemented waste activated sludge in final effluent under different cultivation modes.

Mode and substrate	Lipid (%)	Lipid productivity (mgL ⁻¹ d ⁻¹)	Protein (%)	Protein productivity (mgL ⁻¹ d ⁻¹)	Carbohydrate (%)	Carbohydrate productivity (mgL ⁻¹ d ⁻¹)
Auto BG11	12.7±0.85	10.6±0.00	20.89±0.63	17.4±0.5	28.39±0.25	24.13±0.67
Mixo BG11	15.2±0.99	30.6±0.01	23.15±0.53	46.6±0.1	36.96±0.49	74.4±1.00
Mixo RAW	18.75±4.60	14.93±0.01	26.90±0.08	21.42±.91	19.10±0.01	15.22±0.01
Mixo pH 2 WAS +FE	23.25±1.77	29.89±0.01	18.58±0.06	23.88±0.08	20.83±0.23	26.78±0.29
Mixo pH 2 WAS + FE 250	20.25±1.06	40.86±1.96	19.03±0.05	38.39±0.10	18.09±0.13	36.32±0.26
Mixo WAS + FE 500	22.5±1.41	50.83±2.71	23.06±0.28	52.09±0.64	20.29±0.08	45.70±0.18
Mixo WAS + FE 1500	24.5±0.01	72.95±0.01	24.47±0.15	72.84±0.44	24.55±0.01	73.07±0.02
Hetero BG11	23.7±0.71	54.91±0.01	36±2.61	44.5±0.57	36.50±0.57	77.88±0.38
Hetero RAW	11.75±1.77	1.19±0.01	25.55±0.16	2.59±0.02	16.25±0.16	1.64±0.02
Hetero pH 2 WAS + FE	18.5±0.01	0.93±0.02	24.42±0.08	1.22±0.01	18.82±0.07	0.94±0.01

4.3.5.1. Lipid productivity

The highest lipid content (24.5%) was observed from Mixo WAS + FE 1500, which was found to also have the highest lipid productivity of 72.95±0.01 mgL⁻¹d⁻¹ (Table 4.4 and Figure 4.3). In comparison to Mixo BG11 (15.2% lipid content and 30.6±0.01 mgL⁻¹d⁻¹ lipid productivity). The lipid productivity in Mixo WAS + FE 1500 was more than doubled as compared to lipid productivity in Mixo BG11. Heterotrophic cultivations in BG11 revealed the

lipid content of 23.7% in BG11 coupled with $54.91 \pm 0.01 \text{ mgL}^{-1}\text{d}^{-1}$ lipid productivity. However, under heterotrophic conditions in waste substrate the lipid productivities were extremely low due to low biomass yields. Although the lipid content in Hetero pH 2 WAS + FE (18.5%) was higher than in Mixo BG11 (15.2%) and Auto BG11 (12.7%) the productivity was low ($0.93 \pm 0.02 \text{ mgL}^{-1}\text{d}^{-1}$). Thus for the production of microalgal lipids for industrial applications such as biofuels, Mixo cultivation strategy in WAS + FE 1500 would be preferred over the Hetero conditions.

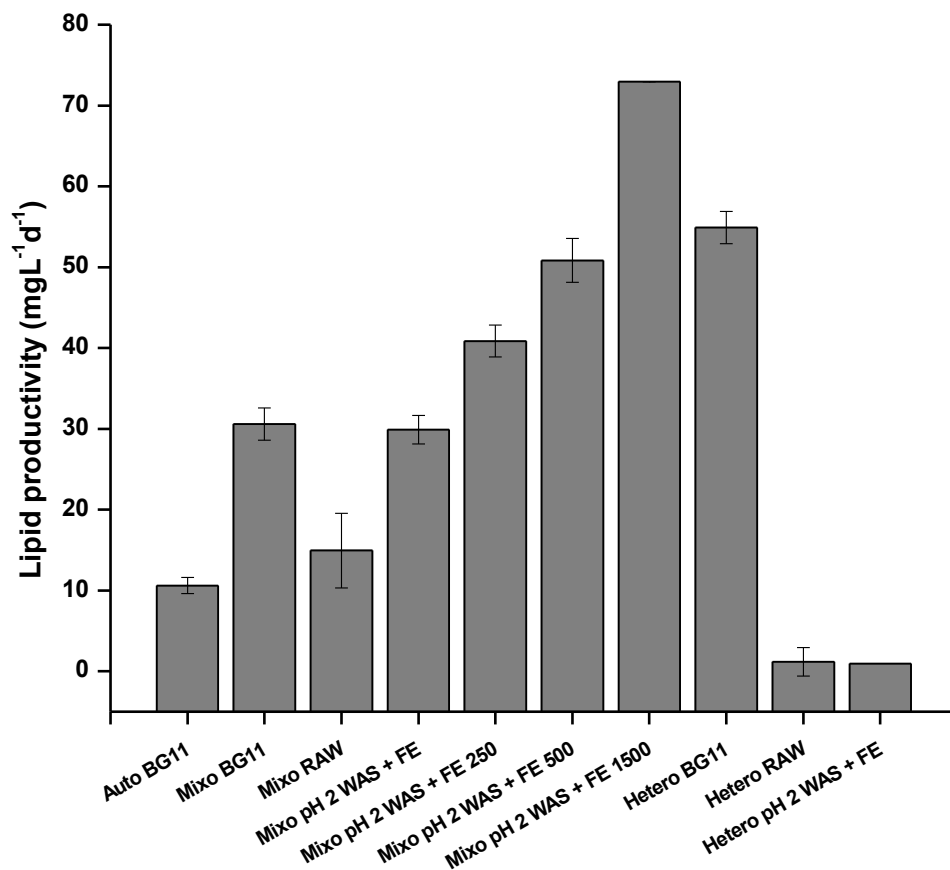


Figure 4.3: Lipid productivities of *Chlorella sorokiniana* in BG11, native and pre-treated waste substrates and waste substrate with urea supplementation, under different cultivation modes. Data represented as mean \pm SD (n=2).

4.3.5.2. Protein productivity

The protein productivities achieved with various experiments in this study are depicted in Figure 4.4. The highest protein productivity was observed from Mixo WAS + FE 1500 ($72.84 \pm 0.44 \text{ mgL}^{-1}\text{d}^{-1}$) followed by Mixo WAS + FE 500 ($52.09 \pm 0.64 \text{ mgL}^{-1}\text{d}^{-1}$) and Mixo BG11 ($46.6 \pm 0.1 \text{ mgL}^{-1}\text{d}^{-1}$). The protein productivities attained from *C. sorokiniana* in urea supplemented Mixo WAS + FE showed excellent results as it surpassed productivities attained from synthetic media. These results were expected as protein synthesis is dependent upon the nitrogen content within the media provided (Uggetti *et al.*, 2014) and in this case urea was added to the waste substrate to exogenously supply N. On the other hand, Hetero conditions were found to be unsuitable for protein production as very low protein productivities of $2.59 \pm 0.02 \text{ mgL}^{-1}\text{d}^{-1}$ and $1.22 \pm 0.01 \text{ mgL}^{-1}\text{d}^{-1}$ were achieved in Hetero RAW and Hetero pH 2 WAS + FE respectively.

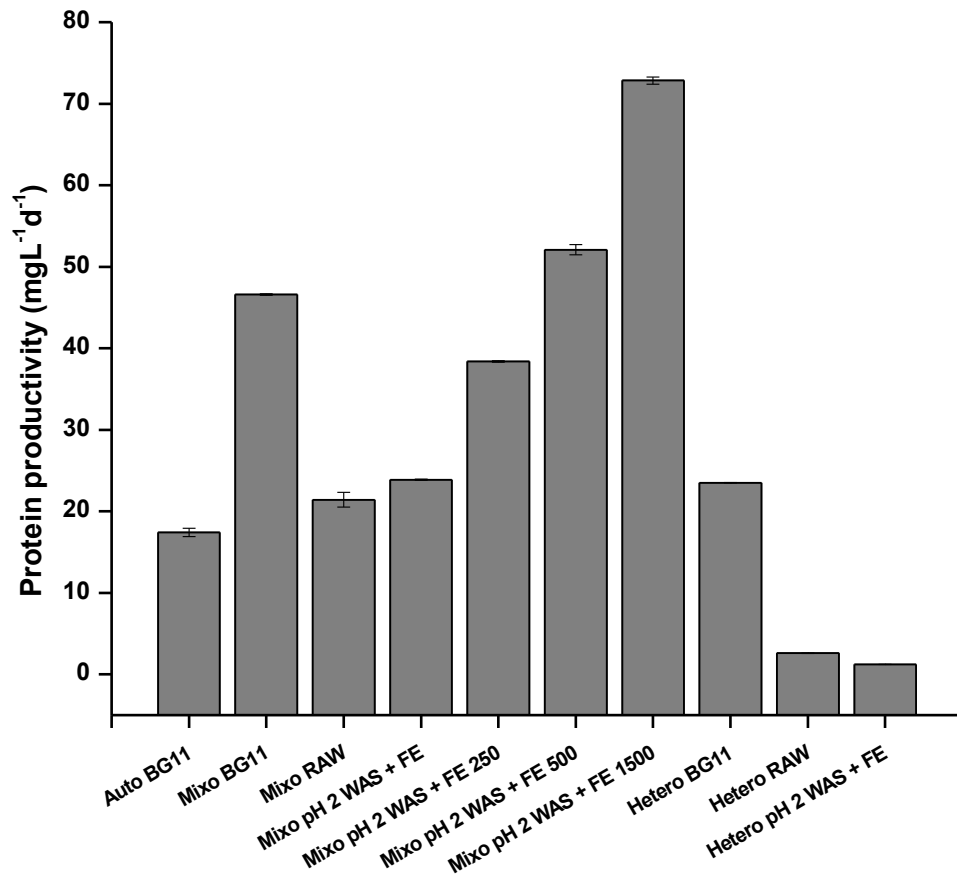


Figure 4.4: Protein productivities of *Chlorella sorokiniana* in BG11, native and pre-treated waste substrates and waste substrate with urea supplementation, under different cultivation modes. Data represented as mean \pm SD (n=2).

It can be deduced that Mixo WAS + FE 500 (20.29% protein content) and Mixo WAS + FE 1500 (24.55% protein content) are promising mode and substrates for microalgae cultivation for protein applications. Ansari *et al.* (2017) observed protein content of 28.81% when *C. sorokiniana* was cultivated in aquaculture wastewater under mixotrophic conditions. In recommendation, the crude protein obtained may be utilized for animal, bird and aquaculture feed. However, extensive research needs to be done to study the suitability of the amino acids from urea supplemented Mixo WAS + FE for this application.

4.3.5.3. Carbohydrate productivity

Apart from lipids and proteins, microalgae biomass have applied value in terms of carbohydrates, specifically as a feedstock for energy and biofuels production such as bioethanol biohydrogen and biogas (Singh *et al.*, 2015). No previous investigations have been put forth to elucidate the suitability of microalgae biomass cultivated in wastewater final effluent with waste activated sludge for carbohydrate applications.

Carbohydrate production abilities differ amongst microalgal species and can be manipulated according to the cultivation conditions provided. Figure 4.5 and Table 4.4 report the carbohydrate contents and productivities attained from *C. sorokiniana* grown in waste substrates in comparison to synthetic media under different cultivation modes. The highest carbohydrate productivities were observed from Hetero BG11 ($77.88 \pm 0.38 \text{ mgL}^{-1}\text{d}^{-1}$) followed by Mixo BG11 ($74.4 \pm 1.00 \text{ mgL}^{-1}\text{d}^{-1}$) and Mixo WAS + FE 1500 ($73.07 \pm 0.02 \text{ mgL}^{-1}\text{d}^{-1}$). Mixotrophic cultivation mode showed decent carbohydrate productivities for urea supplemented waste substrate grown *C. sorokiniana*. However, further investigation needs to be done on the fermentative conversion of hydrolysed microalgae biomass to ethanol from Mixo WAS + FE 1500 cultivation strategy.

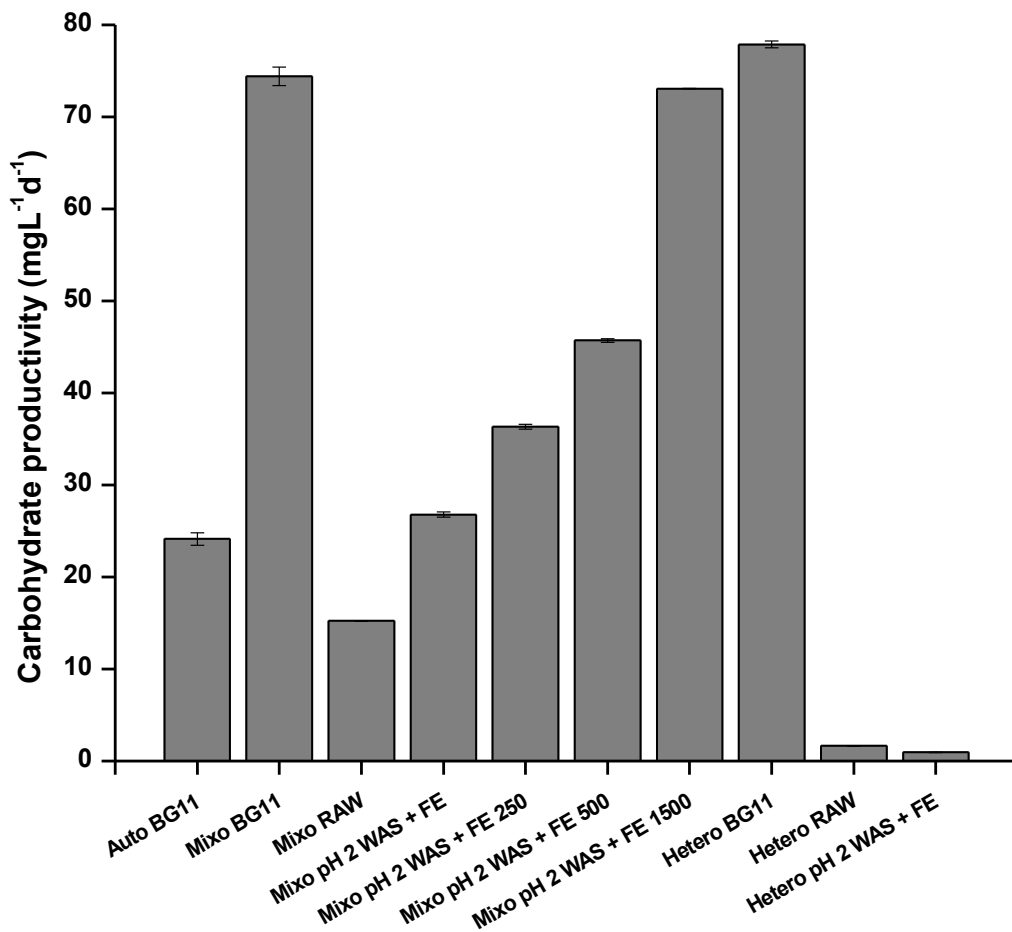


Figure 4.5: Carbohydrate productivities of *Chlorella sorokiniana* in BG11, native and pre-treated waste substrates and waste substrate with urea supplementation, under different cultivation modes. Data represented as mean \pm SD (n=2).

Carbohydrate productivities from heterotrophically grown microalgae ($1.64 \pm 0.02 \text{ mgL}^{-1}\text{d}^{-1}$ in Hetero RAW and $0.94 \pm 0.01 \text{ mgL}^{-1}\text{d}^{-1}$ in Hetero pH 2 WAS + FE) was found to be low for any suitable applications. This could be due to insufficient carbon being provided accompanied by the light limitation and non-photosynthetic metabolism under Hetero conditions as generally in microalgae the accumulation of carbohydrates is the result of CO_2 fixation during

photosynthesis where NADPH and ATP are used to fix CO₂ to produce glucose and sugars through the Calvin cycle pathway (Boyle, 2005).

Table 4.5: Comparison of biomass productivity, metabolites production and nutrients removal by different microalgae grown in substrate derived from solid waste in the literature

Microalgae	Type of wastewater	Pre-treatment	BP* mgL ⁻¹ d ⁻¹	P= productivity in mgL ⁻¹ d ⁻¹			Nutrient removal %			Ref
				C=content in %			NH ₄ -N	NO ₃ -N	PO ₄ -P	
				L*	PR*	CR*				
<i>Chlorella zofingiensis</i>	Olive mill solid waste effluent	2-stage anaerobic digestion and sterilization (121°C, 2 bar pressure, 20 min)	-	-	-	-	-	-	-	(Córdoba <i>et al.</i> , 2008)
<i>Chlamydomonas globosa</i>	Poultry litter extract in industrial wastewater	-	~31	-	-	-	-	-	-	(Bhatnagar <i>et al.</i> , 2011)
<i>Chlorella minutissima</i>	Poultry litter extract in industrial wastewater	-	~41.7	-	-	-	-	-	-	(Bhatnagar <i>et al.</i> , 2011)
<i>Scenedesmus bijuga</i>	Poultry litter extract in industrial wastewater	-	~48.9	-	-	-	-	-	-	(Bhatnagar <i>et al.</i> , 2011)
<i>Chlorella</i> sp.	Liquid fraction of digestate after biogas production from the wastewater sludge and technical glycerol	Filtration	~172	-	-	-	-	-	-	(Skorupskaitė <i>et al.</i> , 2015)
<i>Chlorella</i> sp.	Diluted dairy manure	Anaerobic digestion and filtration	-	-	-	-	100	-	27.4-38.4	(Wang <i>et al.</i> , 2010a)
<i>Chlorella</i> sp.	Swine manure	Acidogenic anaerobic digestion with glucose	276.2	C=26.1	C=58.8	-	-	-	-	(Hu <i>et al.</i> , 2013)
<i>Chlorella sorokiniana</i>	Mixo pH 2 WAS + FE	Acidification (pH 2)	128.6	C=23.3 P=29.9	C=18.6 P=23.9	C=20.8 P=26.8	97.8	60	95	Current study
<i>Chlorella sorokiniana</i>	Mixo WAS + FE 1500	Acidification (pH 2)	298.8	C=24.5 P=73	C=24.5 P=72.8	C=24.6 P=73.1	-	-	-	Current study

*BP- biomass productivity; L- lipid; PR- protein; CR- carbohydrate

Table 4.5 shows the comparison of various solid waste substrates that have been used for the cultivation of microalgae. The previous literature lacks information about the effect of these waste substrates on biochemical composition of microalgal biomass. There have been no reported studies of municipal dried waste activated sludge as a source of nutrients for microalgal cultivation under different cultivation modes. The biomass productivity of $298.75 \pm 55.81 \text{ mgL}^{-1}\text{d}^{-1}$ in Mixo WAS + FE 1500 achieved in this study was higher than the productivities obtained using many other waste substrates (Table 4.5). The developed strategy showed decent lipid, protein and carbohydrate productivities in microalgal biomass for its biofuels and feed based applications. The replacement of synthetic nutrient media and fresh water with waste activated sludge and final effluent makes this microalgal cultivation strategy sustainable and economically feasible for commercial scale biomass production.

4.4. Conclusion

Undoubtedly, waste activated sludge has shown the potential to release nutrients into final effluent to support microalgal growth. Chemical pre-treatment (pH 2 acidification) displayed increased nutrient release from WAS into FE accompanied by significant reduction in bacterial load. Mixotrophic mode of nutrition was found to be most suitable for achieving high biomass productivities. Supplementation of inexpensive N (urea) in WAS + FE gave higher biomass yields compared to synthetic media. Microalgal biomass grown with the developed strategy have shown promising potential for its application for biofuel and feed applications with decent lipid, protein and carbohydrate productivities. The developed strategy thus can replace the synthetic nutrients and fresh water with waste activated sludge and final effluent for economically feasible and sustainable microalgal biomass production.

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1. Significant conclusions of the study

- Physico-chemical characteristics of domestic wastewater revealed that AC and WAS + FE were promising alternatives to synthetic media for the growth and biomass production of *C. sorokiniana*.
- Physical pre-treatment (filtration) was the most feasible in reducing the bacterial load of AC whilst chemical pre-treatment (acidification to pH 2) was the most suitable for nutrient release of WAS into FE and for bacterial reduction.
- Mixotrophic cultivation mode was preferred over heterotrophic cultivation mode when *C. sorokiniana* was cultivated in both AC and WAS + FE with respect to biomass productivities, photosynthetic physiology, efficient N and P removal and metabolites production.
- Due to low nutrient compositions of domestic wastewater, N supplementation was required to promote and sustain microalgal growth.
- Urea supplementation at a concentration of 1500 mgL⁻¹ was the optimum concentration for the enhancement of microalgal biomass productivities, and production of lipids, proteins and carbohydrates in Mixo AC and Mixo WAS + FE.

- Amongst the waste substrates evaluated, the highest biomass and metabolites production was achieved from urea supplemented (1500 mgL⁻¹) Mixo WAS + FE. The physiological responses of *C. sorokiniana* cultivated with Mixo WAS + FE 1500 demonstrated by PAM fluorometry further justifies the efficacy and suitability of this media formulation.
- Biochemical constituents analysis revealed that the metabolites produced from microalgal cultivation in Mixo AC 1500 and Mixo WAS + FE 1500 show immense potential for application in biofuel and feed industries.

5.2. Recommendations

The following are proposed suggestions for enhancing the current research:

- It is recommended that microalgal strains should be investigated individually for their adaptability toward wastewater, different cultivation modes and capabilities for hyper-production of lipid, protein and carbohydrates.
- Health and safety regulations need to be intensely investigated on the utilization of microalgal biomass derived from waste substratum for application in feed, specifically for human consumption.

- The feasibility of other wastewater streams with high N should be investigated for microalgae cultivation.
- This study has been conducted at laboratory-scale thus cultivation challenges that may arise at large-scale have not been examined and cannot be foreseen. Therefore the cultivation strategies investigated need to be performed and optimized at large-scale.
- Interactions (synergistic/ antagonistic) between the desired microalgae and contaminant microorganisms in domestic wastewater should be elucidated to understand the ecosystem and to optimize the cultivation conditions further.

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APPENDICES

APPENDIX A: Ammonia reagents preparation for Thermo Scientific™ Gallery™ automated photometric analyzer (vantaa, finland)

Wastewater samples were first filtered through Whatman no.1 filter paper. Samples (2 mL) were transferred into Gallery sample holders. The instrument was calibrated before samples were analyzed. Upon sample entry, 20 µL of NH₃ Reagent 1 was added to 120 µL of sample. Samples were incubated for 18 s. Following this, 20 µL of NH₃ Reagent 2 was added to the sample and incubated for 600 s. Samples were read at a wavelength of 660 nm.

Principle

Ammonia reacts with hypochlorite ions generated by the alkaline hydrolysis of sodium dichloroisocyanurate to form monochloramine. This reacts with salicylate ions in the presence of sodium nitroprusside at around pH 12.6 to form a blue compound. The absorbance of this compound is measured spectrophotometrically at a wavelength of 660 nm and is related to the NH₃ concentration by means of a calibration curve.

Interference

Magnesium forming a precipitate of Mg hydroxide at high pH values (> 12). The trisodium citrate is used to prevent this interference and the method should tolerate Mg at concentrations normally found in most non-saline waters.

Reagent 1: Sodium Salicylate Solution

65 g of Sodium Salicylate and 65 g of tri-Sodium Citrate was dissolved in 400 mL NH₃ free deionised water, pH was adjusted to 8.0 with 0.4 % Nitric acid. 0.49 g of Sodium Nitroprusside was dissolved in 10 mL of free deionised water. The solution was made up to 500 mL with NH₃ free deionised water. This reagent was stored at 8 °C and had a shelf life of 1 month.

Reagent 2: D.I.C Solution

16 g of sodium hydroxide was dissolved in 250 mL NH₃ free deionised water. It was then cooled. 1.0 g of Sodium Dichloroisocyanurate was dissolved and made up to 500 mL with NH₃ free deionised water. This reagent was stored at 8°C and had a shelf life of 1 month.

Ammonia Standard Solution – 1000 mgL⁻¹ as N

3.819 g of dried NH₄ chloride was dissolved in 1000 mL of NH₃ free water. This solution was stored between 2 – 8 °C and was stable for 1 month.

APPENDIX B: Nitrite reagents preparation for Thermo Scientific™ Gallery™ automated photometric analyzer (vantaa, finland)

Wastewater samples were first filtered through Whatman no.1 filter paper. 2 mL samples were put into Gallery cuvettes. The instrument was calibrated before samples were analyzed. Samples were first incubated for 18 s. 20 µL of TON Reagent 3 was added to the sample and incubated for 360 s. Samples were read at a wavelength of 540 nm.

Principle

Diazotization of sulphanilamide by nitrite in the presence of Phosphoric acid, at 1.9 pH and the subsequent formation of an azo dye with N-1-naphthylethylenediamine (NEDD). The absorbance of this compound is measured spectrophotometrically at 520 nm and is related to the nitrite by means of a calibration curve.

Interference

Oxidising agents, amines, chloramines, thiosulphate, hexametaphosphate, alkalies and ferric iron may cause interferences.

Colour Reagent

50 mL of concentrated Phosphoric acid was carefully added to 500 mL of distilled water. 5 g of sulphanilamide was added and dissolved completely before adding 0.25 g N-(1-naphthyl)-ethylenediaminedihydrochloride. It was then diluted to 1000 mL with distilled water and stored in an amber bottle between 2 - 8 °C. This reagent was stable for 1 month.

Nitrite Standard Solution-100 mgL⁻¹

0.493 g of dried sodium nitrite was dissolved in 1000 mL distilled water.

APPENDIX C: Total Oxidised Nitrogen (TON) reagent preparation for Thermo Scientific™ Gallery™ automated photometric analyzer (vantaa, finland)

Wastewater samples were first filtered through Whatman no.1 paper. 2 mL samples were put into Gallery cuvettes. The instrument was first calibrated, and then read. Upon sample entry, 50 µL of TON Reagent 1 was added to 120 µL of sample. Samples were incubated for 240 s. Following this, 50 µL of TON Reagent 2 was added to the sample and incubated for 420 s. Thereafter, 30 µL of TON Reagent 3 was added to the sample and incubated for 300 s. Samples were read at a wavelength of 660 nm.

Principle

Nitrate is reduced to NO₂ by hydrazine under alkaline conditions. The total NO₂ ions are then reacted with sulphanilamide and N-1-naphthylethylenediamine dihydrochloride under acidic conditions to form a pink azo-dye. The absorbance is measured at 540 nm and is related to the total oxidised N concentration by means of a calibration curve. Nitrate was calculated by subtracting NO₂ from total oxidised N.

Interference

No interferences were identified.

Reagent 1: Sodium Hydroxide

0.8 g sodium hydroxide was dissolved in 100 mL of distilled water. This solution was stable for 1 day.

Reagent 2: Reductant

0.325 g of hydrazine sulphate was dissolved in 400 mL distilled water. 0.75 mL of stock Cu sulphate solution and 5 mL of zinc sulphate was added and made up to 500 mL with distilled water. This solution was stable for 1 month.

Stock solutions required for reductant

Copper sulphate solution

0.78 g Cu sulphate was dissolved in 200 mL of distilled water. This solution was stable for 1 month.

Zinc sulphate solution

9.0 g Zn sulphate was dissolved in 200 mL distilled water. This solution was stable for 1 month.

Reagent 3: Colour reagent

50 mL of concentrated phosphoric acid was carefully added to 500 mL of distilled water. 5 g of sulphanilamide was added and dissolved completely before adding 0.25 g n-(1-naphthyl)-ethylenediaminedihydrochloride. This was diluted to 1000 mL with distilled water and stored in an amber bottle between 2 – 8°C. This solution was stable for 1 month.

Total Oxidised Nitrogen standard solution

1.6306 g of dried KNO_3 was dissolved in a 1000 mL of distilled water. This was stored between 2 - 8°C, this solution was stable for 1 month.

APPENDIX D: Phosphate reagent preparation for Thermo Scientific™ Gallery™ automated photometric analyzer (vantaa, finland)

Principle

Wastewater samples were first filtered through Whatman no.1 filter paper. 2 mL samples were put into Gallery cuvettes. The instrument was calibrated before samples were analyzed. Samples were first incubated for 18 s. 14 µL of Reagent 1 was added to 120 µL of sample and incubated for 120 s. Following this, 6 µL of Reagent 2 was added to the sample and incubated for 540 s. Samples were read at a wavelength of 880 nm.

Interference

Silica forms a pale blue complex which absorbs at 880 nm. This interference is insignificant as to produce a positive 1 mgL⁻¹ error in orthophosphate would require a silica concentration of approximately 4000 mgL⁻¹. The determination is sensitive to variations in acid concentrations, the higher the acidity the lower the sensitivity.

Stock Solutions

Reagent 1: Antimony Potassium Tartrate solution

0.3 g of antimony K tartrate was dissolved in 50 mL distilled water and diluted to 100 mL. This reagent was stored in an amber bottle between 2 – 8°C.

Reagent 2: Ammonium Molybdate

4.0 g of NH_4 molybdate was dissolved in 100 mL distilled water. The reagent was stored in a plastic container.

Reagent 3: Dilute Sulphuric acid

140 mL of concentrated H_2SO_4 was very slowly added to 1000 mL distilled water.

Reagent 4: Ascorbic Acid Solution

1.76 g ascorbic acid was dissolved in 100mL distilled water. This solution was stable for 5 days when refrigerated between 2 – 8°C.

Working Solutions

Reagent 1

75 mL of stock NH_4 Molybdate was added to 250 mL of dilute H_2SO_4 . 25 mL of stock antimony K tartrate was added to this mixture.

Reagent 2

Stock Ascorbic acid solution

28 mL of Reagent 1 was mixed with 12 mL of Reagent 2 for a single shot assay. This was prepared daily.

**APPENDIX E: Tukey's Test for biomass productivities of liquid waste
substrates**

Grouping information using Tukey method

	Mean biomass productivity (mgL ⁻¹ d ⁻¹)	Grouping
Mixo BG11	201.43	A
Hetero BG11	173.81	A
Mixo ADC 1500	162.50	A
Auto BG11	83.18	B
Mixo ADC 500	81.75	B
Mixo ADC	77.14	B
Hetero ADC	76.25	B
Mixo INF	72.50	B
Hetero INF	61.88	B

Means that do not share a letter are significantly different.

**APPENDIX F: Tukey's Test for biomass productivities of solid waste
substrates**

Grouping information using Tukey method

	Mean biomass productivity (mgL ⁻¹ d ⁻¹)	Grouping			
Mixo pH 2 WAS + FE 1500	298.75	A			
Mixo pH 2 WAS + FE 500	225.89	A	B		
Mixo pH 2 WAS + FE 250	201.79	A	B	C	
Mixo BG11	201.43		B	C	
Hetero BG11	173.81		B	C	D
Mixo pH 2 WAS +FE	128.57			C	D
Auto BG11	85			D	E
Mixo RAW	79.64			D	E
Hetero RAW	10.12				E
Hetero pH 2 WAS + FE	5.01				E

Means that do not share a letter are significantly different.

APPENDIX G: Blue-green (BG11) medium

Component	Stock solution (gL ⁻¹)
NaNO ₃	1.5
K ₂ HPO ₄	0.04
MgSO ₄ ·7H ₂ O	0.075
CaCl ₂ ·2H ₂ O	0.036
EDTA (disodium salt)	0.001
Na ₂ CO ₃	0.02
Citric Acid	0.006
Ammonium ferric citrate	0.006
Trace metal solution	1 mL
Trace metal solution	
H ₃ BO ₃	2.86
MnCl ₂ ·4H ₂ O	1.81
ZnSO ₄ ·7H ₂ O	0.22
Na ₂ MoO ₄ ·2H ₂ O	0.39
CuSO ₄ ·5H ₂ O	0.079
Co(NO ₃) ₂ ·6H ₂ O	0.049

The components were added to 1 L⁻¹ distilled water. The pH was adjusted to 7.1 after sterilization (autoclaving). Moreover, 5 gL⁻¹ glucose was added for mixotrophic and heterotrophic cultivations only.

APPENDIX H: Publication: Assessment of municipal wastewaters at various stages of treatment process as potential growth media for *Chlorella sorokiniana* under different modes of cultivation

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Assessment of municipal wastewaters at various stages of treatment process as potential growth media for *Chlorella sorokiniana* under different modes of cultivation



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HIGHLIGHTS

- Anaerobic centrate and influent were suitable growth medium for microalgae.
- Filtration was most feasible pre-treatment for bacterial reduction.
- High biomass productivity found in anaerobic centrate.
- Mixotrophic mode was most suitable cultivation strategy for domestic wastewater.
- Urea supplementation improved biomass and metabolites productivities.

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ABSTRACT

Wastewater utilization for microalgal biomass production is potentially the most economical route for its fuel and feed applications. In this study, suitability of various wastewater streams within a domestic wastewater treatment plant was evaluated for microalgal cultivation. Pre-treatment methods were evaluated to minimize bacterial load. Biomass, cell physiology, nutrient removal efficiencies and biochemical constituents of *Chlorella sorokiniana* were investigated in influent (INF) and anaerobic tank centrate (AC) under mixotrophic (Mixo) and heterotrophic (Hetero) cultivation. Promising biomass ($77.14 \text{ mg L}^{-1} \text{ d}^{-1}$), lipid ($24.91 \text{ mg L}^{-1} \text{ d}^{-1}$), protein ($22.36 \text{ mg L}^{-1} \text{ d}^{-1}$) and carbohydrate ($20.10 \text{ mg L}^{-1} \text{ d}^{-1}$) productivities were observed in Mixo AC with efficient ammonium (94.29%) and phosphate (83.30%) removal. Supplementation of urea at a concentration of 1500 mg L^{-1} further enhanced biomass ($162.50 \text{ mg L}^{-1} \text{ d}^{-1}$), lipid ($24.91 \text{ mg L}^{-1} \text{ d}^{-1}$), protein ($22.36 \text{ mg L}^{-1} \text{ d}^{-1}$) and carbohydrate ($20.10 \text{ mg L}^{-1} \text{ d}^{-1}$) productivities in Mixo AC. Urea supplemented mixotrophic cultivation of microalgae in AC is developed as a biomass production strategy.

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1. Introduction

Microalgal biomass has been put forth as a promising feedstock for biofuels, animal feed, aquaculture, cosmetics, nutraceuticals and biofertilizer industries (Mata et al., 2014). To make these applications sustainable and economical, microalgal biomass needs to be produced on a commercial scale with minimal production cost. Microalgal biomass production is successfully applied on relatively small-scale systems, generally for nutraceuticals and anthropogenic consumption which are high value commodities (Benemann, 2013). The worldwide production of microalgal

biomass does not exceed more than 15 000 tons per annum at a production cost of at least 10 € kg^{-1} (Morales-Amaral et al., 2015). The requirement of large amounts of microalgae-essential macronutrients i.e., nitrogen (N) and phosphorus (P), in addition to carbon dioxide (CO_2), is one of the major reason for high production cost (Morales-Amaral et al., 2015).

In order to alleviate this problem microalgae cultivation using waste materials as opposed to synthetic media has been anticipated, mainly with regard to wastewater reuse and treatment. Wastewater contains a combination of organic matter, nutrients and synthetic compounds. Wastewater contains majority of the nutrients required for microalgal cultivation and thus can be used for biomass production. Discharge of these wastewaters leads to the problem of eutrophication or algal blooms into receiving waters (Morales-Amaral et al., 2015).

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APPENDIX I: Publication: Prospects, recent advancements and challenges of different wastewater streams for microalgal cultivation

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Review

Prospects, recent advancements and challenges of different wastewater streams for microalgal cultivation



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ABSTRACT

Microalgae are recognized as one of the most powerful biotechnology platforms for many value added products including biofuels, bioactive compounds, animal and aquaculture feed etc. However, large scale production of microalgal biomass poses challenges due to the requirements of large amounts of water and nutrients for cultivation. Using wastewater for microalgal cultivation has emerged as a potential cost effective strategy for large scale microalgal biomass production. This approach also offers an efficient means to remove nutrients and metals from wastewater making wastewater treatment sustainable and energy efficient. Therefore, much research has been conducted in the recent years on utilizing various wastewater streams for microalgae cultivation. This review identifies and discusses the opportunities and challenges of different wastewater streams for microalgal cultivation. Many alternative routes for microalgal cultivation have been proposed to tackle some of the challenges that occur during microalgal cultivation in wastewater such as nutrient deficiency, substrate inhibition, toxicity etc. Scope and challenges of microalgal biomass grown on wastewater for various applications are also discussed along with the biorefinery approach.

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APPENDIX J: Publication: Evaluation of waste activated sludge as a potential nutrient source for cultivation of *Chlorella sorokiniana*

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Evaluation of waste activated sludge as a potential nutrient source for cultivation of *Chlorella sorokiniana*



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ABSTRACT

Economical and sustainable microalgal biomass production is crucial for its commercial scale application for energy and other commodities. This work elucidates a novel cultivation strategy where nutrient-rich waste activated sludge (WAS) and final effluent (FE) from a municipal wastewater treatment process is used for microalgal biomass generation. This strategy reduces the use of synthetic nutrients, fertilizers and fresh water. Strategy development included investigation of pre-treatment/extraction methods for effective nutrient release and bacterial load reduction. Evaluation of growth kinetics, photosynthetic performance, nutrient removal efficiencies and biochemical composition of microalgae under mixotrophic (Mixo) and heterotrophic (Hetero) modes of cultivation was performed. Urea supplementation is studied to enhance the biomass productivity. Microalgal cultivation in acid pre-treated WAS + FE with urea supplementation of 1500 mg L⁻¹ showed biomass productivity of 298.75 mg L⁻¹ d⁻¹. Microalgal biomass grown with WAS + FE using developed strategy showed higher lipid and protein productivities and comparable carbohydrate yields to the synthetic media.

1. Introduction

Waste activated sludge (WAS) from the municipal wastewater treatment process is an inevitable by-product. The sludge is generated in large amounts during biological wastewater treatment and is comprised mainly of organic matter, inorganic nutrients and microbial cells. The organic part comprises of approximately 50–55% carbon, 10–15% nitrogen and 1–3% phosphorus [1]. Ash content contains minerals such as microline, calcite or quartz which are formed from elements such as calcium, magnesium, potassium and iron. Moreover, certain heavy metals such as chromium, nickel, copper, zinc, lead cadmium and mercury can also be found in the sludge [2]. Following biological treatment, the sludge is dried and disposed of on landfill sites or sacrificial lands [3]. Approximately 40–60% of wastewater treatment plant's expenses are directly related to WAS treatment and disposal [4].

Due to its favourable nutrient composition and when stabilized through a composting process, WAS has been successfully utilized as organic fertilizers or soil additives. The WAS comes to the forefront of microalgal technology as it could be used as a potential nutrient source for microalgal cultivation. Utilizing WAS as a nutrient source for microalgal cultures could minimize the dependence on synthetic fertilizers reducing overall cost of production. Use of final effluent as a liquid medium will minimize the use of fresh water. Overall, this could be a sustainable waste management strategy which provides environmental

benefit in terms of re-cycling of nutrients, primarily nitrogen (N) and phosphorous (P), from sludge and final effluent by incorporation into microalgal biomass. Moreover, this technique may offer environmental benefits in terms of nutrient recovery from the sludge, whilst achieving microalgal biomass with potential applications in fuel and feed industries.

Currently there is no research published on the utilization of municipal dried waste activated sludge as a nutrient medium for microalgal biomass production and its applied value. There have been very few reports on the utilization of solid wastes with high nutrient concentrations for microalgal cultivation. Pleissner et al. [5] heterotrophically cultivated *Schizochytrium mangrovei* and *Chlorella pyrenoidosa* using nutrient-rich fungal hydrolysed food waste. Researchers have also explored different animal wastes (poultry and pig) as growth medium for microalgae [6]. Most studies focus on liquid waste substrates for microalgae cultivation and have only implemented photoautotrophic cultivation strategies with the primary aim of being lipid production and nutrient removal. There are existing research gaps that are required to be addressed. These include identifying a suitable waste nutrient source, pre-treatment/extraction methods for the reduction of bacterial contaminants as well as effective nutrient release from waste source or biosolids into the medium, exploring various microalgal cultivation modes and attaining biomass productivities comparable to synthetic media and biochemical composition analysis of biomass for its

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